



(Document name)

Specification

(Title of the invention)

GLYCOPROTEIN PRODUCTION CELL

(Scope of the claims)

(Claim 1) A cell in which activity of an enzyme relating to modification of a sugar chain of a glycoprotein is decreased by an artificial technique.

(Claim 2) The cell according to claim 1, wherein the enzyme relating to modification of a sugar chain of a glycoprotein is an enzyme relating to modification of a sugar chain reducing end of a glycoprotein.

(Claim 3) A cell in which activity of an enzyme relating to modification of a sugar chain reducing end of a glycoprotein is controlled by an artificial technique.

(Claim 4) A cell in which activity of an enzyme relating to modification of a sugar chain reducing end of a glycoprotein is increased by an artificial technique.

(Claim 5) The cell according to any one of claims 1 to 4, wherein the artificial technique is selected from the group consisting of the following (a), (b), (c) (d) and (e):

(a) a technique for adding an inhibitor or activator for the activity of the enzyme to a medium;

(b) a technique for selecting a mutant relating to the enzyme;

(c) a technique for introducing a gene encoding the enzyme;

(d) a technique of a gene disruption for targeting a gene encoding the enzyme;

(e) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

(Claim 6) The cell according to claim 5, wherein the technique selecting a mutant is a technique in which a desired mutant is selected as a measure of the activity of

the enzyme or the sugar chain structure of the glycoprotein among naturally occurring mutants or mutants obtained by subjecting the parent strain to a mutation-inducing treatment.

(Claim 7) The cell according to claim 6, the technique for selecting a desired mutant as a measure of the sugar chain structure is a technique for selecting a desired mutant as a measure of the presence or absence of a sugar bound to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 8) The cell according to claim 7, wherein the sugar to be bound is fucose.

(Claim 9) The cell according to claim 5, wherein the technique for a gene disruption is an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a gene disruption technique using a transposon.

(Claim 10) The cell according to any one of claims 1 to 9, wherein the enzyme is glycosyltransferase.

(Claim 11) The cell according to claim 10, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(Claim 12) The cell according to claim 10, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(Claim 13) The cell according to any one of claims 10 to 12, wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 14) The cell according to claim 13, wherein the sugar to be bound is fucose.

(Claim 15) The cell according to any one of claims 10 to 14, wherein the glycosyltransferase is fucosyltransferase.

(Claim 16) The cell according to any one of claims 10 to 15, wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.

(Claim 17) A cell to which a DNA selected from the group consisting of the following (a), (b), (c) and (d) is introduced so that an activity of a protein encoded by the DNA is increased:

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having an activity of a protein encoded by the DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(d) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having an activity of a protein encoded by the DNA comprising the nucleotide sequence represented by SEQ ID NO:2.

(Claim 18) A cell in which a technique for a gene disruption as a measure of a genome DNA selected from the group consisting of the following (a), (b) and (c) is carried out so that an activity of a protein encoded by an exon on the genome DNA is inhibited:

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) an anisogenome DNA corresponding to a synteny of genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions.

(Claim 19) The cell according to claim 18, wherein the technique for a gene disruption is an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a gene disruption technique using a transposon.

(Claim 20) The cell according to any one of claims 1 to 19, which is a cell selected from the group consisting of a bacterium, a yeast, an animal cell, an insect cell and a plant cell.

(Claim 21) The cell according to claim 20, wherein the animal cell is selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h) and (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line, YB2/3HL.P2.G11.16Ag.20 cell;

(c) a mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

(Claim 22) The cell according to any one of claims 1 to 21, wherein the glycoprotein is an immunologically functional molecule.

(Claim 23) The cell according to any one of claims 1 to 22, which is capable of producing an immunologically functional molecule having a higher effector function than

an immunologically functional molecule produced by the parent strain.

(Claim 24) The cell according to claim 23, wherein the immunologically functional molecule having a higher effector function is an immunologically functional molecule comprising a sugar chain in which a sugar is not bound to *N*-acetylglucosamine in a reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 25) The cell according to any one of claims 1 to 22, which is capable of producing an immunologically functional molecule having a lower effector function than an immunologically functional molecule produced by the parent strain.

(Claim 26) The cell according to claim 25, wherein the immunologically functional molecule having a lower effector function is an immunologically functional molecule comprising a sugar chain in which a sugar is not bound to *N*-acetylglucosamine in a reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 27) The cell according to claim 24 or 26, wherein the sugar to be bound is fucose.

(Claim 28) The cell according to any one of claims 23 to 27, wherein the effector function is a cytotoxic activity through an Fc region.

(Claim 29) The cell according to claim 28, wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(Claim 30) The cell according to any one of claims 1 to 29, to which a gene encoding an immunologically functional molecule is introduced.

(Claim 31) The cell according to any one of claims 22 to 30, wherein the immunologically functional molecule is a protein or a peptide.

(Claim 32) The cell according to claim 31, wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(Claim 33) The cell according to claim 32, wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(Claim 34) The cell according to claim 33, wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor

receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(Claim 35) A transgenic non-human animal or plant or the progenies thereof, comprising a genome which is modified such that the activity of an enzyme relating to modification of a sugar chain of a glycoprotein is controlled.

(Claim 36) The transgenic non-human animal or plant or the progenies thereof according to claim 35, which is a knock out animal or plant.

(Claim 37) The transgenic non-human animal or plant or the progenies thereof according to claim 35, which is prepared by using the embryonic stem cell or the fertilized egg cell according to claim 21.

(Claim 38) The transgenic non-human animal or plant or the progenies thereof according to any one of claims 35 to 37, wherein the transgenic non-human animal is selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(Claim 39) The transgenic non-human animal or plant or the progenies thereof according to any one of claims 35 to 38, wherein the enzyme relating to modification of a sugar chain of a glycoprotein is glycosyltransferase.

(Claim 40) The transgenic non-human animal or plant or the progenies thereof according to claim 39, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(Claim 41) The transgenic non-human animal or plant or the progenies thereof according to claim 39, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(Claim 42) The transgenic non-human animal or plant or the progenies thereof according to any one of claims 39 to 41, wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to N-acetylglucosamine in the reducing end of an N-glycosyl-linked sugar chain.

(Claim 43) The transgenic non-human animal or plant or the progenies thereof according to claim 42, wherein the fucose to be bound is fucose.

(Claim 44) The transgenic non-human animal or plant or the progenies thereof according to any one of claims 39 to 43, wherein the glycosyltransferase is fucosyltransferase.

(Claim 45) The transgenic non-human animal or plant or the progenies thereof according to any one of claims 39 to 43, wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.

(Claim 46) A method for producing an immunologically functional molecule, which comprises culturing the cell according to any one of claims 1 to 34 to produce and accumulate the immunologically functional molecule in the culture; and purifying the immunologically functional molecule from the culture.

(Claim 47) A method for producing an immunologically functional molecule, which comprises transplanting the cell according to any one of claims 1 to 34 into the living body; rearing the transplanted living body; isolating an individual, tissue or body fluid comprising an objective substance from the transplanted

living body; and purifying the immunologically functional molecule from the isolated individual, tissue or body fluid.

(Claim 48) The method according to claim 46 or 47, which produces an immunologically functional molecule having a higher effector function than an immunologically functional molecule produced by a cell which is not subjected to an artificial technique.

(Claim 49) The method according to claim 46 or 47, which produces an immunologically functional molecule having a lower effector function than an immunologically functional molecule produced by a cell which is not subjected to an artificial technique.

(Claim 50) A method for producing an immunologically functional molecule, which comprises rearing the transgenic non-human animal or plant or the progenies thereof according to any one of claims 35 to 45; isolating tissue or body fluid comprising an objective substance from the animal or plant; and purifying the immunologically functional molecule from the isolated tissue or body fluid.

(Claim 51) The method according to claim 50, which produces an immunologically functional molecule having a higher effector function than a transgenic non-human animal or plant or the progenies thereof comprising a genome which is not modified.

(Claim 52) The method according to claim 50, which produces an immunologically functional molecule having a lower effector function than a transgenic non-human animal or plant or the progenies thereof comprising a genome which is not modified.

(Claim 53) The method according to any one of claims 48 to 52, wherein the effector function is a cytotoxic activity through an Fc region.

(Claim 54) The method according to claim 53, wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(Claim 55) The method according to any one of claims 46 to 54, wherein the immunologically functional molecule is a protein or a peptide.

(Claim 56) The method according to claim 55, wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(Claim 57) The method according to claim 56, wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(Claim 58) The cell according to claim 57, wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis

factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(Claim 59) An immunologically functional molecule produced by the method according to any one of claims 46 to 58.

(Claim 60) An immunologically functional molecule composition, comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain, wherein among the total *N*-glycoside-linked sugar chains in the composition, the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition is 20% or more.

(Claim 61) An immunologically functional molecule composition, comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain, wherein among the total *N*-glycoside-linked sugar chains in the composition, the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition is 10% or less.

(Claim 62) The immunologically functional molecule composition according to claim 60, which is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain

in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain and has a higher effector function than the original immunologically functional molecule composition.

(Claim 63) The immunologically functional molecule composition according to claim 62, wherein the higher effector function is 10 times or more than the effector function of the original immunologically functional molecule.

(Claim 64) The immunologically functional molecule composition according to claim 61, which is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain and has a lower effector function than the original immunologically functional molecule composition.

(Claim 65) The immunologically functional molecule composition according to claim 64, wherein the lower effector function is 1/10 times or less than the effector function of the original immunologically functional molecule.

(Claim 66) The immunologically functional molecule composition according to any one of claims 62 to 65, wherein the effector function is a cytotoxic activity through an Fc region.

(Claim 67) The immunologically functional molecule composition according to claim 66, wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(Claim 68) The immunologically functional molecule composition according to any one of claims 60 to 67, wherein the *N*-glycoside-linked sugar chain is a sugar chain selected from the group consisting of the following (a), (b) and (c):

(a) a high mannose type sugar chain;

(b) a complex type sugar chain;

(c) a hybrid type sugar chain.

(Claim 69) The immunologically functional molecule composition according to any one of claims 60 to 68, wherein the sugar which is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(Claim 70) The immunologically functional molecule composition according to any one of claims 60 to 69, which is produced by the steps of culturing a cell which is capable of producing an immunologically functional molecule in a medium to produce and accumulate the immunologically functional molecule in the culture; and purifying an objective composition from the culture.

(Claim 71) The immunologically functional molecule composition according to claim 70, wherein the cell is selected from the group consisting of a bacterium, a yeast, an animal cell, an insect cell and a plant cell.

(Claim 72) The immunologically functional molecule composition according to claim 71, wherein the animal cell is selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h) and (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line, YB2/3HL.P2.G11.16Ag.20 cell;

(c) a mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

(Claim 73) A method for producing the immunologically functional molecule composition according to any one of claims 60 to 69, which comprises rearing a transgenic non-human animal or plant or the progenies thereof; isolating tissue or body fluid comprising an objective substance from the animal or plant; and purifying the objective substance from the isolated tissue or body fluid.

(Claim 74) The immunologically functional molecule composition according to claim 73, wherein the transgenic non-human animal or plant or the progenies is a knock out animal or plant.

(Claim 75) The immunologically functional molecule composition according to claim 73 or 74, wherein the transgenic non-human animal is a transgenic non-human animal prepared by using the embryonic stem cell or the fertilized egg cell according to claim 21.

(Claim 76) The immunologically functional molecule composition according to any one of claims 73 to 75, wherein the transgenic non-human animal is selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(Claim 77) The immunologically functional molecule composition according to any one of claims 60 to 76, wherein the immunologically functional molecule is a protein or a peptide.

(Claim 78) The immunologically functional molecule composition according to claim 77, wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(Claim 79) The immunologically functional molecule composition according to claim 78, wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-

related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(Claim 80) The immunologically functional molecule composition according to claim 79, wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(Claim 81) A medicament comprising, as an active ingredient, comprising the immunologically functional molecule according to claim 59 or the immunologically functional molecule composition according to any one of claims 60 to 80.

(Claim 82) The medicament according to claim 81, which is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(Claim 83) A method for preparing a medicament having a desired effector function, which comprises changing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain.

(Claim 84) The method according to claim 83, wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a higher effector function than the original medicament.

(Claim 85) The method according to claim 84, wherein the higher effector function is 10 times or more than the effector function of the original medicament.

(Claim 86) The method according to claim 84 or 85, wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the

composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 20% or more of the total *N*-glycoside-linked sugar chains in the composition.

(Claim 87) The method according to claim 83, wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a lower effector function than the original medicament.

(Claim 88) The method according to claim 87, wherein the lower effector function is 1/10 times or less than the effector function of the original medicament.

(Claim 89) The method according to claim 87 or 88, wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 10% or less of the total *N*-glycoside-linked sugar chains in the composition.

(Claim 90) The method according to any one of claims 83 to 89, wherein the effector function is a cytotoxic activity through an Fc region.

(Claim 91) The method according to claim 90, wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(Claim 92) The method according to any one of claims 83 to 91, wherein the *N*-glycoside-linked sugar chain is a sugar chain selected from the group consisting of the following (a), (b) and (c):

(a) a high mannose type sugar chain;

- (b) a complex type sugar chain;
- (c) a hybrid type sugar chain.

(Claim 93) The method according to any one of claims 83 to 92, wherein the sugar which is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(Claim 94) A medicament which is prepared by the method of any one of claims 83 to 93.

(Claim 95) The medicament according to claim 94, which is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(Claim 96) A method for preparing a cell in which activity of an enzyme relating to modification of a sugar chain of a glycoprotein is controlled, which comprises selecting or modifying a cell by the artificial technique selected from the group consisting of (a) to (e) according to claim 5; and selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(Claim 97) A method for screening a cell in which an activity of an enzyme relating to modification of a glycoprotein is controlled, which comprises selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(Claim 98) The method according to claim 96 or 97, wherein said selecting of a desired cell as a measure of a sugar chain structure of a glycoprotein is carried out by

selecting a desired cell as a measure of the presence or absence of a sugar bound to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 99) The method according to claim 98, wherein the sugar to be bound is fucose.

(Claim 100) The method according to any one of claims 96 to 99, wherein the enzyme relating to modification of a glycoprotein is glycosyltransferase.

(Claim 101) The method according to claim 100, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(Claim 102) The method according to claim 100, wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(Claim 103) The method according to any one of claims 100 to 102, wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(Claim 104) The method according to claim 103, wherein the sugar to be bound is fucose.

(Claim 105) The method according to any one of claims 100 to 105, wherein the glycosyltransferase is fucosyltransferase.

(Claim 106) The method according to any one of claims 100 to 105, wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.

(Claim 107) The method according to any one of claims 96 to 106, wherein the glycoprotein is an immunologically functional molecule.

(Claim 108) The method according to claim 107, wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in

which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(Claim 109) The method according to claim 107, wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in which fucose is bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(Claim 110) The method according to claim 108 or 109, wherein the sugar to be bound is fucose.

(Claim 111) The method according to any one of claims 107 to 110, wherein the immunologically functional molecule is a protein or a peptide.

(Claim 112) The method according to claim 111, wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(Claim 113) The method according to claim 112, wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(Claim 114) The method according to claim 113, wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth

factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(Claim 115) A DNA comprising the nucleotide sequence represented by SEQ ID NO:1 in the Sequence Listings.

(Claim 116) A DNA comprising the nucleotide sequence represented by SEQ ID NO:2 in the Sequence Listings.

(Claim 117) A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) an anisogenome DNA corresponding to a synteny of genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions.

(Claim 118) A target vector comprising the DNA according to any one of claims 115 to 117.

(Detailed description of the invention)

(0001)

(Technical field of the invention)

The present invention relates to a cell for the production of a glycoprotein such as an immunologically functional molecule or the like, e.g., an antibody useful for various diseases, a fragment of the antibody and a fusion protein having the Fc region of the antibody, a peptide, etc., a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule, and use thereof.

(0002)

(Background art)

Since antibodies have high binding activity, binding specificity and high stability in blood, their applications to diagnosis, prevention and treatment of various human diseases have been attempted [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1995)]. Also, production of a humanized antibody such as a human chimeric antibody or a human complementarity determining region (hereinafter referred to as "CDR")-grafted antibody from an antibody derived from an animal other than human have been attempted by using genetic recombination techniques. The human chimeric antibody is an antibody in which its antibody variable region (hereinafter referred to as "V region") is an antibody derived from an animal other than human and its constant region (hereinafter referred to as "C region") is derived from a human antibody. The human CDR-grafted antibody is an antibody in which the CDR of a human antibody is replaced by CDR of an antibody derived from an animal other than human.

(0003)

It has been revealed that five classes, namely IgM, IgD, IgG, IgA and IgE, are present in antibodies derived from mammals. Antibodies of human IgG class are mainly used for the diagnosis, prevention and treatment of various human diseases because they have functional characteristics such as long half-life in blood, various effector functions and the like [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 1 (1995)]. The human IgG class antibody is further classified into the following 4 subclasses: IgG1, IgG2, IgG3 and IgG4. A large number of studies have so far been conducted for antibody-dependent cell-mediated cytotoxic activity (hereinafter referred to as "ADCC activity") and complement-dependent cytotoxic activity (hereinafter referred to as "CDC activity") as effector functions of the IgG class antibody, and it has been reported that among antibodies of the human IgG class, the IgG1 subclass has the highest ADCC activity and CDC activity [*Chemical Immunology*, 65, 88 (1997)]. In view of the above, most of the anti-tumor humanized antibodies, including commercially available Rituxan and Herceptin, which require high effector functions for the expression of their effects, are antibodies of the human IgG1 subclass.

(0004)

Expression of ADCC activity and CDC activity of the human IgG1 subclass antibodies requires binding of the Fc region of the antibody to an antibody receptor existing on the surface of an effector cell, such as a killer cell, a natural killer cell, an activated macrophage or the like (hereinafter referred to as "FcγR") and various complement components are bound. Regarding the binding, it has been suggested that several amino acid residues in the hinge region and the second domain of C region (hereinafter referred to as "Cγ2 domain") of the antibody are important [*Eur. J. Immunol.*, 23, 1098 (1993), *Immunology*, 86, 319

(1995), *Chemical Immunology*, 65, 88 (1997)] and that a sugar chain binding to the C $\gamma$ 2 domain [*Chemical Immunology*, 65, 88 (1997)] is also important.

(0005)

Regarding the sugar chain, Boyd et al. have examined effects of a sugar chain on the ADCC activity and CDC activity by treating a human CDR-grafted antibody CAMPATH-1H (human IgG1 subclass) produced by a Chinese hamster ovary cell (CHO cell) or a mouse myeloma NSO cell (NSO cell) with various sugar hydrolyzing enzymes, and reported that elimination of the non-reducing end sialic acid did not have influence upon both activities, but the CDC activity alone was affected by further removal of galactose residue and about 50% of the activity was decreased, and that complete removal of the sugar chain caused disappearance of both activities [*Molecular Immunol.*, 32, 1311 (1995)]. Also, Lifely et al. have analyzed the sugar chain bound to a human CDR-grafted antibody CAMPATH-1H (human IgG1 subclass) which was produced by CHO cell, NSO cell or rat myeloma YO cell, measured its ADCC activity, and reported that the CAMPATH-1H derived from YO cell showed the highest ADCC activity, suggesting that N-acetylglucosamine (hereinafter referred also to as "GlcNAc") at the bisecting position is important for the activity [*Glycobiology*, 5, 813 (1995); WO 99/54342]. These reports indicate that the structure of the sugar chain plays an important role in the effector functions of human antibodies of IgG1 subclass and that it is possible to prepare an antibody having more higher effector function by changing the structure of the sugar chain. However, actually, structures of sugar chains are various and complex, and it cannot be said that an actual important structure for the effector function was identified.

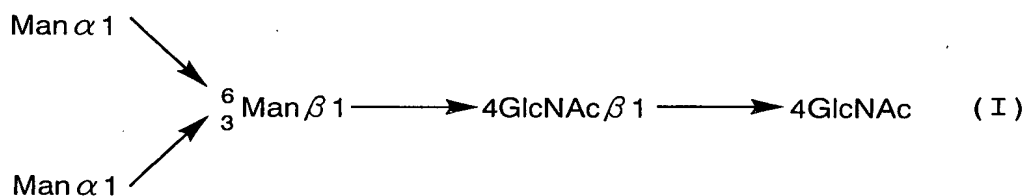
(0006)

Sugar chains of glycoproteins are roughly divided into two types, namely a sugar chain which binds to asparagine (*N*-glycoside-linked sugar chain) and a sugar chain which binds to other amino acid such as serine, threonine (*O*-glycoside-linked sugar chain), based on the binding form to the protein moiety. The *N*-glycoside-linked sugar chains have various structures [*Biochemical Experimentation Method 23 - Method for Studying Glycoprotein Sugar Chain* (Gakujutsu Shuppan Center), edited by Reiko Takahashi (1989)], but it is known that they have a basic common core structure shown by the following structural formula (I).

(0007)

(Chem. 1)

Formula (I)



(0008)

The sugar chain terminus which binds to asparagine is called a reducing end, and the opposite side is called a non-reducing end. It is known that the *N*-glycoside-linked sugar chain includes a high mannose type in which mannose alone binds to the non-reducing end of the core structure; a complex type in which the non-reducing end side of the core structure has at least one parallel branches of galactose-*N*-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and the non-reducing end side of Gal-GlcNAc has a structure of sialic acid, bisecting *N*-acetylglucosamine or the like; a hybrid type in which the non-reducing end side of the core structure has branches of

both of the high mannose type and complex type; and the like.

(0009)

In the Fc region of an antibody of an IgG type, two N-glycoside-linked sugar chain binding sites are present. In serum IgG, to the sugar chain binding site, generally, binds a complex type sugar chain having plural branches and in which addition of sialic acid or bisecting N-acetylglucosamine is low. It is known that there is variety regarding the addition of galactose to the non-reducing end of the complex type sugar chain and the addition of fucose to the N-acetylglucosamine in the reducing end [*Biochemistry*, 36, 130 (1997)].

(0010)

It has been considered that such a structure of a sugar chain is determined by sugar chain genes, namely a gene for a glycosyltransferase which synthesizes a sugar chain and a gene for a glycolytic enzyme which hydrolyzes the sugar chain.

(0011)

Synthesis of an N-glycoside-linked sugar chain is described below.

Glycoproteins are modified with a sugar chain in the endoplasmic reticulum (hereinafter referred to as "ER") lumen. During the biosynthesis step of the N-glycoside-linked sugar chain, a relatively large sugar chain is transferred to the polypeptide chain which is elongating in the ER lumen. In the transformation, the sugar chain is firstly added in succession to phosphate groups of a long chain lipid carrier comprising about 20  $\alpha$ -isoprene units, which is called dolichol phosphate (hereinafter referred also to as "P-Dol"). That is, N-acetylglucosamine is transferred to dolichol phosphate to thereby form GlcNAc-P-P-Dol and then one more GlcNAc is transferred to form GlcNAc-GlcNAc-P-P-Dol. Next, five mannoses (hereinafter

mannose is also referred to as "Man") are transferred to thereby form  $(\text{Man})_5-(\text{GlcNAc})_2\text{-P-P-Dol}$  and then four Man's and three glucoses (hereinafter glucose is also referred to as "Glc") are transferred. Thus, a sugar chain precursor,  $(\text{Glc})_3-(\text{Man})_9-(\text{GlcNAc})_2\text{-P-P-Dol}$ , called core oligosaccharide is formed. The sugar chain precursor comprising 14 sugars is transferred as a mass to a polypeptide having an asparagine-X-serine or asparagine-X-threonine sequence in the ER lumen. In the reaction, dolichol pyrophosphate (P-P-Dol) bound to the core oligosaccharide is released but again becomes dolichol phosphate by hydrolysis with pyrophosphatase and is recycled. Trimming of the sugar chain immediately starts after the sugar chain binds to the polypeptide. That is, 3 Glc's and 1 or 2 Man's are eliminated on the ER, and it is known that  $\alpha$ -1,2-glucosidase I,  $\alpha$ -1,3-glucosidase II and  $\alpha$ -1,2-mannosidase relates to the elimination.

(0012)

The glycoprotein which was subjected to trimming on the ER is transferred to the Golgi body and are variously modified. In the *cis* part of the Golgi body, *N*-acetylglucosamine phosphotransferase which relates to addition of mannose phosphate, *N*-acetylglucosamine 1-phosphodiester  $\alpha$ -*N*-acetylglucosaminidase and  $\alpha$ -mannosidase I are present and reduce the Man residues to 5. In the medium part of the Golgi body, *N*-acetylglucosamine transferase I (GnTI) which relates to addition of the first outside GlcNAc of the complex type *N*-glycoside-linked sugar chain,  $\alpha$ -mannosidase II which relates to elimination of 2 Man's, *N*-acetylglucosamine transferase II (GnTII) which relates to addition of the second GlcNAc from the outside and  $\alpha$ -1,6-fucosyltransferase which relates to addition of fucose to the reducing end *N*-acetylglucosamine are present. In the *trans* part of the Golgi body, galactose transferase which relates to addition

of galactose and sialyltransferase which relates to addition of sialic acid such as *N*-acetylneuraminic acid or the like are present. It is known that *N*-glycoside-linked sugar chain is formed by activities of these various enzymes.

(0013)

In general, most of the humanized antibodies of which application to medicaments is in consideration are prepared using genetic recombination techniques and produced using Chinese hamster ovary tissue-derived CHO cell as the host cell. But as described above, since the sugar chain structure plays a remarkably important role in the effector function of antibodies and differences are observed in the sugar chain structure of glycoproteins expressed by host cells, development of a host cell which can be used for the production of an antibody having higher effector function is desired.

In order to modify the sugar chain structure of the produced glycoprotein, various methods have been attempted, such as 1) application of an inhibitor against an enzyme relating to the modification of a sugar chain, 2) selection of a mutant, 3) introduction of a gene encoding an enzyme relating to the modification of a sugar chain, and the like. Specific examples are described below.

(0014)

Examples of an inhibitor against an enzyme relating to the modification of a sugar chain include tunicamycin which selectively inhibits formation of GlcNAc-P-P-Dol which is the first step of the formation of a core oligosaccharide which is a precursor of an *N*-glycoside-linked sugar chain, castanospermin and *N*-methyl-1-deoxynojirimycin which are inhibitors of glycosidase I, bromocondulitol which is an inhibitor of glycosidase II, 1-deoxynojirimycin and 1,4-dioxy-1,4-imino-D-mannitol which are inhibitors of mannosidase I, swainsonine which is an

inhibitor of mannosidase II and the like. Examples of an inhibitor specific for a glycosyltransferase include deoxy derivatives of substrates against N-acetylglucosamine transferase V (GnTV) and the like [*Glycobiology Series 2 - Destiny of Sugar Chain in Cell* (Kodan-sha Scientific), edited by Katsutaka Nagai, Senichiro Hakomori and Akira Kobata (1993)]. Also, it is known that 1-deoxynojirimycin inhibits synthesis of a complex type sugar chain and increases the ratio of high mannose type and hybrid type sugar chains. Actually, it has been reported that sugar chain structure of IgG was changed and properties such as antigen binding activity and the like was changed when the inhibitors were added to a medium [*Molecular Immunol.*, 26, 1113 (1989)].

(0015)

Mutants regarding the activity of an enzyme relating to the modification of a sugar chain are mainly selected and obtained as a lectin-resistant cell line. For example, CHO cell mutants having various sugar chain structures have been obtained as a lectin-resistant cell line using a lectin such as WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (concanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*) or the like [*Somatic Cell Mol. Genet.*, 12, 51 (1986)].

(0016)

As an example of the modification of the sugar chain structure of a product obtained by introducing the gene of an enzyme relating to the modification of a sugar chain into a host cell, it has been reported that a protein in which a number of sialic acid is added to the non-reducing end of the sugar chain can be produced by

introducing rat  $\beta$ -galactoside- $\alpha$ -2,6-sialyltransferase into CHO cell [*J. Biol. Chem.*, 261, 13848 (1989)].

Also, it was confirmed that an H antigen (Fuc $\alpha$ 1-2Gal $\beta$ 1-) in which fucose (hereinafter also referred to as "Fuc") was added to the non-reducing end of the sugar chain was expressed by introducing human  $\beta$ -galactoside-2- $\alpha$ -fucosyltransferase into mouse L cell [*Science*, 252, 1668 (1991)]. In addition, based on knowledge that addition of the bisecting-positioned N-acetylglucosamine of N-glycoside-linked sugar chain is important for the ADCC activity of antibody, Umana et al. have prepared CHO cell which expresses  $\beta$ -1,4-N-acetylglucosamine transferase III (GnTIII) and compared it with the parent cell line on the expression of GnTIII. It was confirmed that expression of GnTIII was not observed in the parent cell line of CHO cell [*J. Biol. Chem.*, 261, 13370 (1984)], and that the antibody expressed using the produced GnTIII expressing CHO cell had ADCC activity 16 times higher than the antibody expressed using the parent cell line [*Glycobiology*, 5, 813 (1995): WO 99/54342]. At this time, Umana et al. have also produced CHO cell into which  $\beta$ -1,4-N-acetylglucosamine transferase V (GnTV) was introduced and reported that excess expression of GnTIII or GnTV shows toxicity for CHO cell.

(0017)

Thus, in order to modify the sugar chain structure of the glycoprotein to be produced, control of the activity of the enzyme relating to the modification of a sugar chain in the host cell has been attempted, but actually, the structures of sugar chains are various and complex, and solution of the physiological roles of sugar chains would be insufficient, so that trial and error are repeated. Particularly, although it has been revealed little by little that the effector function of antibodies is greatly influenced by the sugar chain structure, a truly important sugar chain structure has not been specified yet.

Accordingly, identification of a sugar chain which has influence upon the effector function of antibodies and development of a host cell to which such a sugar chain structure can be added are expected for developing medicaments.

(0018)

(Problems to be solved by the invention)

An object of the present invention is to provide a cell useful as a host cell for the production of a glycoprotein which can control the sugar chain structure of the glycoprotein; especially, a cell capable of producing an immunologically functional molecule of which effector function is controlled, a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule produced by the production method, and their use.

(0019)

(Means to solve the problem)

A host cell into which an  $\alpha$ -1,6-fucosyltransferase gene was artificially introduced was prepared so that an antibody having a lower ADCC activity in comparison with the case where the parent strain was used as a host strain could be successfully obtained. Also, a host cell in which an  $\alpha$ -1,6-fucosyltransferase gene was artificially disrupted was prepared so that an antibody having a higher ADCC activity in comparison with the case where the parent strain was used as the host cell. Based on these knowledge, intensive studies were carried out and thus the present invention has been accomplished.

(0020)

The present invention relates to the following (1) to (118):

(1) A cell in which activity of an enzyme relating to modification of a sugar chain of a glycoprotein is decreased by an artificial technique.

(2) The cell according to (1), wherein the enzyme relating to modification of a sugar chain of a glycoprotein is an enzyme relating to modification of a sugar chain reducing end of a glycoprotein.

(3) A cell in which activity of an enzyme relating to modification of a sugar chain reducing end of a glycoprotein is controlled by an artificial technique.

(4) A cell in which activity of an enzyme relating to modification of a sugar chain reducing end of a glycoprotein is increased by an artificial technique.

(0021)

(5) The cell according to any one of (1) to (4), wherein the artificial technique is selected from the group consisting of the following (a), (b), (c) (d) and (e):

(a) a technique for adding an inhibitor or activator for the activity of the enzyme to a medium;

(b) a technique for selecting a mutant relating to the enzyme;

(c) a technique for introducing a gene encoding the enzyme;

(d) a technique of a gene disruption for targeting a gene encoding the enzyme;

(e) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

(6) The cell according to (5), wherein the technique selecting a mutant is a technique in which a desired mutant is selected as a measure of the activity of the enzyme or the sugar chain structure of the glycoprotein among naturally occurring mutants or mutants obtained by subjecting the parent strain to a mutation-inducing treatment.

(0022)

(7) The cell according to (6), the technique for selecting a desired mutant as a measure of the sugar chain structure is a technique for selecting a desired mutant as

a measure of the presence or absence of a sugar bound to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(8) The cell according to (7), wherein the sugar to be bound is fucose.

(9) The cell according to (5), wherein the technique for a gene disruption is an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a gene disruption technique using a transposon.

(0023)

(10) The cell according to any one of (1) to (9), wherein the enzyme is glycosyltransferase.

(11) The cell according to (10), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(12) The cell according to (10), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(13) The cell according to any one of (10) to (12), wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(14) The cell according to (13), wherein the sugar to be bound is fucose.

(15) The cell according to any one of (10) to (14), wherein the glycosyltransferase is fucosyltransferase.

(16) The cell according to any one of (10) to (15), wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.

(17) A cell to which a DNA selected from the group consisting of the following (a), (b), (c) and (d) is introduced so that an activity of a protein encoded by the DNA is increased:

(a) a DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(b) a DNA comprising the nucleotide sequence represented by SEQ ID NO:2;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions and encodes a protein having an activity of a protein encoded by the DNA comprising the nucleotide sequence represented by SEQ ID NO:1;

(d) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions and encodes a protein having an activity of a protein encoded by the DNA comprising the nucleotide sequence represented by SEQ ID NO:2.

(0024)

(18) A cell in which a technique for a gene disruption as a measure of a genome DNA selected from the group consisting of the following (a), (b) and (c) is carried out so that an activity of a protein encoded by an exon on the genome DNA is inhibited:

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) an anisogenome DNA corresponding to a synteny of genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions.

(19) The cell according to (18), wherein the technique for a gene disruption is an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a gene disruption technique using a transposon.

(20) The cell according to any one of (1) to (19), which is a cell selected from the group consisting of a

bacterium, a yeast, an animal cell, an insect cell and a plant cell.

(21) The cell according to (20), wherein the animal cell is selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h) and (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line, YB2/3HL.P2.G11.16Ag.20 cell;

(c) a mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

(0025)

(22) The cell according to any one of (1) to (21), wherein the glycoprotein is an immunologically functional molecule.

(23) The cell according to any one of (1) to (22), which is capable of producing an immunologically functional molecule having a higher effector function than an immunologically functional molecule produced by the parent strain.

(24) The cell according to (23), wherein the immunologically functional molecule having a higher effector function is an immunologically functional molecule comprising a sugar chain in which a sugar is not bound to N-acetylglucosamine in a reducing end of an N-glycosyl-linked sugar chain.

(25) The cell according to any one of (1) to (22), which is capable of producing an immunologically functional molecule having a lower effector function than an

immunologically functional molecule produced by the parent strain.

(26) The cell according to (25), wherein the immunologically functional molecule having a lower effector function is an immunologically functional molecule comprising a sugar chain in which a sugar is not bound to *N*-acetylglucosamine in a reducing end of an *N*-glycosyl-linked sugar chain.

(27) The cell according to (24) or (26), wherein the sugar to be bound is fucose.

(28) The cell according to any one of (23) to (27), wherein the effector function is a cytotoxic activity through an Fc region.

(29) The cell according to (28), wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(30) The cell according to any one of (1) to (29), to which a gene encoding an immunologically functional molecule is introduced.

(31) The cell according to any one of (22) to (30), wherein the immunologically functional molecule is a protein or a peptide.

(0026)

(32) The cell according to (31), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(33) The cell according to (32), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(34) The cell according to (33), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(35) A transgenic non-human animal or plant or the progenies thereof, comprising a genome which is modified such that the activity of an enzyme relating to

modification of a sugar chain of a glycoprotein is controlled.

(36) The transgenic non-human animal or plant or the progenies thereof according to (35), which is a knock out animal or plant.

(37) The transgenic non-human animal or plant or the progenies thereof according to (35), which is prepared by using the embryonic stem cell or the fertilized egg cell according to (21).

(0027)

(38) The transgenic non-human animal or plant or the progenies thereof according to any one of (35) to (37), wherein the transgenic non-human animal is selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(39) The transgenic non-human animal or plant or the progenies thereof according to any one of (35) to (38), wherein the enzyme relating to modification of a sugar chain of a glycoprotein is glycosyltransferase.

(40) The transgenic non-human animal or plant or the progenies thereof according to (39), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(41) The transgenic non-human animal or plant or the progenies thereof according to (39), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(42) The transgenic non-human animal or plant or the progenies thereof according to any one of (39) to (41), wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(43) The transgenic non-human animal or plant or the progenies thereof according to (42), wherein the fucose to be bound is fucose.

(44) The transgenic non-human animal or plant or the progenies thereof according to any one of (39) to (43), wherein the glycosyltransferase is fucosyltransferase.

(45) The transgenic non-human animal or plant or the progenies thereof according to any one of (39) to (43), wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.  
(0028)

(46) A method for producing an immunologically functional molecule, which comprises culturing the cell according to any one of (1) to (34) to produce and accumulate the immunologically functional molecule in the culture; and purifying the immunologically functional molecule from the culture.

(47) A method for producing an immunologically functional molecule, which comprises transplanting the cell according to any one of (1) to (34) into the living body; rearing the transplanted living body; isolating an individual, tissue or body fluid comprising an objective substance from the transplanted living body; and purifying the immunologically functional molecule from the isolated individual, tissue or body fluid.

(48) The method according to (46) or (47), which produces an immunologically functional molecule having a higher effector function than an immunologically functional molecule produced by a cell which is not subjected to an artificial technique.

(49) The method according to (46) or (47), which produces an immunologically functional molecule having a lower effector function than an immunologically functional molecule produced by a cell which is not subjected to an artificial technique.

(50) A method for producing an immunologically functional molecule, which comprises rearing the transgenic non-human animal or plant or the progenies thereof according to any one of (35) to (45); isolating tissue or body fluid comprising an objective substance from the animal or plant; and purifying the immunologically functional molecule from the isolated tissue or body fluid.

(51) The method according to (50), which produces an immunologically functional molecule having a higher effector function than a transgenic non-human animal or plant or the progenies thereof comprising a genome which is not modified.

(52) The method according to (50), which produces an immunologically functional molecule having a lower effector function than a transgenic non-human animal or plant or the progenies thereof comprising a genome which is not modified.

(0029)

(53) The method according to any one of (48) to (52), wherein the effector function is a cytotoxic activity through an Fc region.

(54) The method according to (53), wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(55) The method according to any one of (46) to (54), wherein the immunologically functional molecule is a protein or a peptide.

(56) The method according to (55), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(57) The method according to (56), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody

which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(58) The cell according to (57), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(0030)

(59) An immunologically functional molecule produced by the method according to any one of (46) to (58).

(60) An immunologically functional molecule composition, comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain, wherein among the total *N*-glycoside-linked sugar chains in the composition, the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition is 20% or more.

(61) An immunologically functional molecule composition, comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain, wherein among the total *N*-glycoside-linked sugar chains in the composition, the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition is 10% or less.

(62) The immunologically functional molecule composition according to (60), which is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain and has a higher effector function than the original immunologically functional molecule composition.

(63) The immunologically functional molecule composition according to (62), wherein the higher effector function is 10 times or more than the effector function of the original immunologically functional molecule.

(0031)

(64) The immunologically functional molecule composition according to (61), which is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain and has

a lower effector function than the original immunologically functional molecule composition.

(65) The immunologically functional molecule composition according to (64), wherein the lower effector function is 1/10 times or less than the effector function of the original immunologically functional molecule.

(66) The immunologically functional molecule composition according to any one of (62) to (65), wherein the effector function is a cytotoxic activity through an Fc region.

(67) The immunologically functional molecule composition according to (66), wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(68) The immunologically functional molecule composition according to any one of (60) to (67), wherein the *N*-glycoside-linked sugar chain is a sugar chain selected from the group consisting of the following (a), (b) and (c):

- (a) a high mannose type sugar chain;
- (b) a complex type sugar chain;
- (c) a hybrid type sugar chain.

(69) The immunologically functional molecule composition according to any one of (60) to (68), wherein the sugar which is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(70) The immunologically functional molecule composition according to any one of (60) to (69), which is produced by the steps of culturing a cell which is capable of producing an immunologically functional molecule in a medium to produce and accumulate the immunologically

functional molecule in the culture; and purifying an objective composition from the culture.

(71) The immunologically functional molecule composition according to (70), wherein the cell is selected from the group consisting of a bacterium, a yeast, an animal cell, an insect cell and a plant cell.

(0032)

(72) The immunologically functional molecule composition according to (71), wherein the animal cell is selected from the group consisting of the following (a), (b), (c), (d), (e), (f), (g), (h) and (i):

(a) a CHO cell derived from a Chinese hamster ovary tissue;

(b) a rat myeloma cell line, YB2/3HL.P2.G11.16Ag.20 cell;

(c) a mouse myeloma cell line, NSO cell;

(d) a mouse myeloma cell line, SP2/0-Ag14 cell;

(e) a BHK cell derived from a syrian hamster kidney tissue;

(f) an antibody-producing hybridoma cell;

(g) a human leukemia cell line Namalwa cell;

(h) an embryonic stem cell;

(i) a fertilized egg cell.

(73) A method for producing the immunologically functional molecule composition according to any one of (60) to (69), which comprises rearing a transgenic non-human animal or plant or the progenies thereof; isolating tissue or body fluid comprising an objective substance from the animal or plant; and purifying the objective substance from the isolated tissue or body fluid.

(74) The immunologically functional molecule composition according to (73), wherein the transgenic non-human animal or plant or the progenies is a knock out animal or plant.

(75) The immunologically functional molecule composition according to (73) or (74), wherein the transgenic non-human animal is a transgenic non-human animal prepared by using the embryonic stem cell or the fertilized egg cell according to (21).

(76) The immunologically functional molecule composition according to any one of (73) to (75), wherein the transgenic non-human animal is selected from the group consisting of cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey and rabbit.

(77) The immunologically functional molecule composition according to any one of (60) to (76), wherein the immunologically functional molecule is a protein or a peptide.

(78) The immunologically functional molecule composition according to (77), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(0033)

(79) The immunologically functional molecule composition according to (78), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(80) The immunologically functional molecule composition according to (79), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor

receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody, or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(81) A medicament comprising, as an active ingredient, comprising the immunologically functional molecule according to (59) or the immunologically functional molecule composition according to any one of (60) to (80).

(82) The medicament according to (81), which is a diagnostic drug, a preventive drug or a therapeutic drug for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(83) A method for preparing a medicament having a desired effector function, which comprises changing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain.

(0034)

(84) The method according to (83), wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a higher effector function than the original medicament.

(85) The method according to (84), wherein the higher effector function is 10 times or more than the effector function of the original medicament.

(86) The method according to (84) or (85), wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 20% or more of the total *N*-glycoside-linked sugar chains in the composition.

(87) The method according to (83), wherein the medicament having a desired effector function is obtained by increasing the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in a composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain and has a lower effector function than the original medicament.

(88) The method according to (87), wherein the lower effector function is 1/10 times or less than the effector function of the original medicament.

(89) The method according to (87) or (88), wherein the ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain is controlled to be 10% or less of the total *N*-glycoside-linked sugar chains in the composition.

(0035)

(90) The method according to any one of (83) to (89), wherein the effector function is a cytotoxic activity through an Fc region.

(91) The method according to (90), wherein the cytotoxic activity is an antibody-dependent cell-mediated cytotoxic activity.

(92) The method according to any one of (83) to (91), wherein the *N*-glycoside-linked sugar chain is a sugar chain selected from the group consisting of the following (a), (b) and (c):

- (a) a high mannose type sugar chain;
- (b) a complex type sugar chain;
- (c) a hybrid type sugar chain.

(93) The method according to any one of (83) to (92), wherein the sugar which is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain is a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(94) A medicament which is prepared by the method of any one of (83) to (93).

(95) The medicament according to (94), which is a diagnostic drug, a preventive drug or a therapeutic drug

for diseases accompanied by tumors, diseases accompanied by allergies, diseases accompanied by inflammations, autoimmune diseases, circulatory organ diseases, diseases accompanied by viral infections or diseases accompanied by bacterial infections.

(96) A method for preparing a cell in which activity of an enzyme relating to modification of a sugar chain of a glycoprotein is controlled, which comprises selecting or modifying a cell by the artificial technique selected from the group consisting of (a) to (e) according to (5; and selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(0036)

(97) A method for screening a cell in which an activity of an enzyme relating to modification of a glycoprotein is controlled, which comprises selecting a desired cell as a measure of an activity of an enzyme relating to modification of a sugar chain of a glycoprotein or a sugar chain structure of a glycoprotein.

(98) The method according to (96) or (97), wherein said selecting of a desired cell as a measure of a sugar chain structure of a glycoprotein is carried out by selecting a desired cell as a measure of the presence or absence of a sugar bound to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(99) The method according to (98), wherein the sugar to be bound is fucose.

(100) The method according to any one of (96) to (99), wherein the enzyme relating to modification of a glycoprotein is glycosyltransferase.

(101) The method according to (100), wherein the glycosyltransferase is glycosyltransferase having no

influence on growth of a cell comprising the glycosyltransferase by decrease of the activity thereof.

(102) The method according to (100), wherein the glycosyltransferase is glycosyltransferase having no influence on growth of a cell comprising the glycosyltransferase by increase of the activity thereof.

(103) The method according to any one of (100) to (102), wherein the glycosyltransferase is an enzyme having an activity of binding a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycosyl-linked sugar chain.

(104) The method according to (103), wherein the sugar to be bound is fucose.

(105) The method according to any one of (100) to (105), wherein the glycosyltransferase is fucosyltransferase.

(0037)

(106) The method according to any one of (100) to (105), wherein the glycosyltransferase is  $\alpha$ -1,6-fucosyltransferase.

(107) The method according to any one of (96) to (106), wherein the glycoprotein is an immunologically functional molecule.

(108) The method according to (107), wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in which fucose is not bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(109) The method according to (107), wherein the immunologically functional molecule is an immunologically functional molecule having a sugar chain in which fucose is bound to *N*-acetylglucosamine in the reducing end of the *N*-glycoside-linked sugar chain.

(110) The method according to (108) or (109), wherein the sugar to be bound is fucose.

(111) The method according to any one of (107) to (110), wherein the immunologically functional molecule is a protein or a peptide.

(112) The method according to (111), wherein the protein is an antibody, an antibody fragment, or a fusion protein comprising an Fc region.

(0038)

(113) The method according to (112), wherein the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen, or an antibody which recognizes a viral or bacterial infection-related antigen.

(114) The method according to (113), wherein the antibody which recognizes a tumor-related antigen is an anti-GD2 antibody, an anti-GD3 antibody, an anti-GM2 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-MAGE antibody, an anti-HM1.24 antibody, an anti-parathyroid hormone-related protein (PTHrP) antibody, an anti-basic fibroblast growth factor antibody, an anti-basic fibroblast growth factor receptor antibody, an anti-FGF8 antibody, an anti-FGF8 receptor antibody, an anti-insulin-like growth factor antibody, an anti-PMSA antibody, an anti-vascular endothelial cell growth factor antibody, or an anti-vascular endothelial cell growth factor receptor antibody; the antibody which recognizes an allergy- or inflammation-related antigen is an anti-interleukin 6 antibody, an anti-interleukin 6 receptor antibody, an anti-interleukin 5 antibody, an anti-interleukin 5 receptor antibody, an anti-interleukin 4 antibody, an anti-interleukin 4 receptor antibody, an anti-tumor necrosis factor antibody, an anti-tumor necrosis factor receptor antibody, an anti-CCR4 antibody, an anti-chemokine antibody,

or an anti-chemokine receptor antibody; the antibody which recognizes a circulatory organ disease-related antigen an anti-GpIIb/IIIa antibody, an anti-platelet-derived growth factor antibody, an anti-platelet-derived growth factor receptor antibody, or an anti-blood coagulation factor antibody; the antibody which recognizes an autoimmune disease-related antigen is an anti-self-DNA antibody; and the antibody which recognizes a viral or bacterial infection-related antigen is an anti-gp120 antibody, an anti-CD4 antibody, an anti-CCR4 antibody or an anti-Vero toxin antibody.

(115) A DNA comprising the nucleotide sequence represented by SEQ ID NO:1 in the Sequence Listings.

(116) A DNA comprising the nucleotide sequence represented by SEQ ID NO:2 in the Sequence Listings.

(117) A genome DNA selected from the group consisting of the following (a), (b) and (c):

(a) a genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(b) an anisogenome DNA corresponding to a synteny of genome DNA comprising the nucleotide sequence represented by SEQ ID NO:3;

(c) a DNA which hybridizes with the DNA comprising the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions.

(118) A target vector comprising the DNA according to any one of (115 to 117).

(0039)

(Embodiment for carrying out the invention)

In the present invention, an enzyme relating to the modification of a sugar chain of a glycoprotein may be any enzyme so long as it can influence the addition of a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycoside-linked sugar chain. The expression "to influence the addition of a sugar" means that the addition of the

sugar is directly or indirectly inhibited or accelerated. Herein, the expression "the addition of the sugar is indirectly inhibited or accelerated" means that the enzyme activity of an enzyme which directly inhibits or accelerates the addition of a sugar (e.g., fucosyltransferase) is influenced or a sugar chain structure as a substrate is influenced.

Examples include  $\alpha$ -1,2-glucosidase I,  $\alpha$ -1,3-glucosidase II,  $\alpha$ -1,2-mannosidase, N-acetylglucosamine phosphotransferase, N-acetylglucosamine-1-phosphodiester- $\alpha$ -N-acetylglucosaminidase,  $\alpha$ -mannosidase I, N-acetylglucosamine transferase (GnTI),  $\alpha$ -mannosidase II, N-acetylglucosamine transferase (GnTII), galactose transferase, sialic acid transferase, N-acetylglucosamine transferase III (GnTIII), N-acetylglucosamine transferase V (GnTV), FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9 (with regard to FUTs 1-9, see *Protein, Nucleic Acid, Enzyme*, extra number, 43(16), 2394 (Dec., 1994) and the like. Among these, fucosyltransferases such as FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9 and the like are preferred, and  $\alpha$ -1,6-fucosyltransferase is more preferred.

(0040)

In the present invention, the method which controls the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein by an artificial technique may be any method, so long as it can artificially control the activity of an enzyme which influences the addition of a sugar to N-acetylglucosamine in the reducing end of an N-glycoside-linked sugar chain.

(0041)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is controlled by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein,

i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is decreased or increased in comparison with the parent strain. In the glycoprotein expressed by the cell, a difference of the modification state of a sugar to *N*-acetylglucosamine in the reducing end of an *N*-glycoside-linked sugar chain is found in comparison with a glycoprotein expressed by the parent cell.

(0042)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is decreased by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is decreased in comparison with the parent strain or deleted.

(0043)

In the present invention, the cell in which the activity of an enzyme relating to the modification of a sugar chain is increased by an artificial technique includes a cell in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, i.e., the activity of an enzyme which influences the addition of a sugar chain to *N*-acetylglucosamine in the reducing end of *N*-glycoside-linked sugar chain, is increased in comparison with the parent strain.

(0044)

The parent strain in the present invention may be any cell line, so long as the activity of an enzyme relating to the modification of is not changed by an artificial technique.

The sugar which is bound to *N*-acetylglucosamine in the reducing end of an *N*-acetylglucosamine in the present invention may be any sugar, so long as the effect of the present invention can be obtained by the sugar. Examples of the sugar include troise, tetrose, pentose, hexsose, heptose and the like, and hexsoses are preferred. Also, examples of the hexsose include glucose, galactose, mannose, fucose and the like, and fucose is particularly preferred.

(0045)

In the present invention, the artificial technique includes

(a) a technique for adding an inhibitor or activator for the activity of the enzyme to a medium;

(b) a technique for selecting a mutant relating to the enzyme;

(c) a technique for introducing a gene encoding the enzyme;

(d) a technique of a gene disruption for targeting a gene encoding the enzyme; and

(e) a technique for inhibiting transcription and/or translation of a gene encoding the enzyme.

(0046)

In the present invention, the glycoprotein may be any one, but a complex protein having a sugar chain structure other than proteoglycan is preferred, and an immunologically functional molecule is more preferred.

The immunologically functional molecule may be any molecule, so long as it related to various immunological reactions in the living body. Specifically, it includes a protein, a peptide and the like. The protein and the peptide include an antibody, an antibody fragment, a fusion protein comprising an Fc region, and the like.

(0047)

As the present invention, the sugar chain of a glycoprotein, an *N*-glycoside-linked sugar chain is



long as the effect of the present invention is obtained from the composition. The term "structurally" means that the sugar chain structures binding to the Fc region is different.

(0050)

In the present invention, when the activity of an enzyme relating to the modification in the reducing end of an *N*-glycoside-linked sugar chain of the immunologically functional molecule is decreased, it is preferred that the effector function of the immunologically functional molecule is higher than that prior to the treatment of the artificially technique.

On the other hand, in the present invention, when the activity of an enzyme relating to the modification in the reducing end of an *N*-glycoside-linked sugar chain of the immunologically functional molecule is increased, it is preferred that the effector function of the immunologically functional molecule is lower than that prior to the treatment of the artificially technique.

In the present invention, the immunologically functional molecule composition comprises a structurally single immunologically functional molecule or structurally plural immunologically functional molecules (so-called mixture).

(0051)

In the present invention, the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the composition comprising an immunologically functional molecule having an *N*-acetylglucosamine in the reducing end in the composition is a ratio of the number of the *N*-glycoside-linked sugar chains in which a sugar is not bound to *N*-acetylglucosamine in the composition against the total number of the *N*-glycoside-linked sugar chains in which a sugar is bound to *N*-acetylglucosamine and the *N*-glycoside-linked sugar chains

in which a sugar is bound to *N*-acetylglucosamine in the composition.

The ratio of a sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the immunologically functional molecule composition is preferably 20% or more, more preferably 30% or more, still more preferably 50% or more, far preferably 80% or more, particularly preferably 90% or more, and the most preferably 100%. The immunologically functional molecule composition has a higher effector function than an immunologically functional molecule composition having lower ratio of *N*-glycoside-linked sugar chain to which a sugar is not bound to *N*-acetylglucosamine in the reducing end.

(0052)

For example, when the immunologically functional molecule composition is an immunologically functional molecule prepared from a cell in which the activity of an enzyme is decreased by the artificial technique, the higher effector function means that the cytotoxic activity is at least two times or more, preferably 5 times or more, more preferably 10 times or more, and most preferably 100 times or more, higher than that in the immunologically functional molecule prepared from the parent strain at an antibody concentration of 0.00001 to 10  $\mu\text{g/ml}$ .

(0053)

In the present invention, the immunologically functional molecule composition having a ratio of *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the immunologically functional molecule composition has a lower effector function than the immunologically functional molecule composition having a high rate of *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end.

(0054)

For example, when the immunologically functional molecule composition is an immunologically functional molecule prepared from a cell in which the activity of an enzyme is increased by the artificial technique, the lower effector function means that the cytotoxic activity is at least 1/2 times or less, preferably 1/5 times or less, more preferably 1/10 times or less, and most preferably 1/100 times or less, lower than that in the immunologically functional molecule prepared from the parent strain at an antibody concentration of 0.00001 to 10 µg/ml.

(0055)

The ratio of an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the reducing end in the composition comprising an immunologically functional molecule having an *N*-glycoside-linked sugar chain can be determined by releasing the sugar chain from the antibody molecule using a known method such as hydrazinolysis, enzyme digestion or the like [Biochemical Experimentation Methods 23 - Method for Studying Glycoprotein Sugar Chain (Japan Scientific Societies Press), edited by Reiko Takahashi (1989)], carrying out fluorescence labeling or radioisotope labeling of the released sugar chain and then separating the labeled sugar chain by chromatography. Also, the released sugar chain can also be determined by analyzing it with the HPAED-PAD method [*J. Liq. Chromatogr.*, 6, 1557 (1983)].

(0056)

In the present invention, the antibody is a protein which is produced in the living body by immune reaction as a result of exogenous antigen stimulation and has an activity to specifically bind to the antigen. Examples of the antibody include an antibody secreted by a hybridoma cell prepared from a spleen cell of an animal immunized with an antigen; an antibody prepared by a genetic

recombination technique, namely an antibody obtained by introducing an antibody gene-inserted antibody expression vector into a host cell; and the like. Specific examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

(0057)

A hybridoma is a cell which is obtained by cell fusion between a B cell obtained by immunizing a mammal other than human with an antigen and a myeloma cell derived from mouse or the like and can produce a monoclonal antibody having the desired antigen specificity.

Examples of the humanized antibody include a human chimeric antibody, a human complementarity determining region (CDR)-grafted antibody and the like.

A human chimeric antibody is an antibody which comprises an antibody heavy chain variable region (hereinafter referred to as "HV" or "VH", the heavy chain being "H chain") and an antibody light chain variable region (hereinafter referred to as "LV" or "VL", the light chain being "L chain"), both of an animal other than human, a human antibody heavy chain constant region (hereinafter also referred to as "CH") and a human antibody light chain constant region (hereinafter also referred to as "CL"). As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used, so long as a hybridoma can be prepared therefrom.

(0058)

The human chimeric antibody can be produced by obtaining cDNA's encoding VH and VL from a monoclonal antibody-producing hybridoma, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a human chimeric antibody expression vector, and then introducing the vector into a host cell to express the antibody.

(0059)

As the CH of human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg") can be used. But those belonging to the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the  $\kappa$  class or  $\lambda$  class can also be used.

A human CDR-grafted antibody is an antibody in which amino acid sequences of CDR's of VH and VL of an antibody derived from an animal other than human are grafted into appropriate positions of VH and VL of a human antibody.

The human CDR-grafted antibody can be produced by constructing cDNA's encoding V regions in which CDR's of VH and VL of an antibody derived from an animal other than human are grafted into CDR's of VH and VL of a human antibody, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a human CDR-grafted antibody expression vector, and then introducing the expression vector into a host cell to express the human CDR-grafted antibody.

(0060)

As the CH of human CDR-grafted antibody, any CH can be used, so long as it belongs to the hIg, but those of the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human CDR-grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the  $\kappa$  class or  $\lambda$  class can also be used.

(0061)

A human antibody is originally an antibody naturally existing in the human body, but it also includes antibodies obtained from a human antibody phage library, a human antibody-producing transgenic animal and a human antibody-producing transgenic plant, which are prepared based on the recent advance in genetic engineering, cell engineering and developmental engineering techniques.

Regarding the antibody existing in the human body, a lymphocyte capable of producing the antibody can be cultured by isolating a human peripheral blood lymphocyte, immortalizing it by its infection with EB virus or the like and then cloning it, and the antibody can be purified from the culture.

(0062)

The human antibody phage library is a library in which antibody fragments such as Fab, single chain antibody and the like are expressed on the phage surface by inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the marker. The antibody fragment can be converted further into a human antibody molecule comprising two full H chains and two full L chains by genetic engineering techniques.

(0063)

A human antibody-producing transgenic non-human animal is an animal in which a human antibody gene is introduced into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a human antibody gene into ES cell of a mouse, transplanting the ES cell into an early stage embryo of other mouse and then developing it. By introducing a human chimeric antibody gene into a fertilized egg and developing it, the transgenic animal can be also prepared. Regarding

the preparation method of a human antibody from the human antibody-producing transgenic animal, the human antibody can be produced and accumulated in a culture by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in mammals other than human and then culturing it.

Examples of the transgenic non-human animal include cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit and the like.

(0064)

Also, in the present invention, it is preferable that the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen or an antibody which recognizes a viral or bacterial infection-related antigen, and a human antibody which belongs to the IgG class is preferable.

(0065)

Examples of the antibody which recognizes a tumor-related antigen include anti-GD2 antibody (Ohta et al., *Anticancer Res.*, 13, 331-336, 1993), anti-GD3 antibody (Ohta et al., *Cancer Immunol. Immunother.*, 36, 260-266, 1993), anti-GM2 antibody (Nakamura et al., *Cancer Res.*, 54, 1511-1516, 1994), anti-HER2 antibody (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-CD52 antibody (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89, 4285-4289, 1992), anti-MAGE antibody (Jungbluth et al., *British J. Cancer*, 83, 493-497, 2000), anti-HM1.24 antibody (Ono et al., *Molecular Immunol.*, 36, 387-395, 1999), anti-parathyroid hormone-related protein (PTHrP) antibody (Ogata et al., *Cancer*, 88, 2909-2911, 2000), anti-basic fibroblast growth factor antibody and anti-FGF8 antibody (Matsuzaki et al., *Proc. Natl. Acad. Sci. USA*, 86, 9911-9915, 1989),

anti-basic fibroblast growth factor receptor antibody and anti-FGF8 receptor antibody (Kuo et al., *J. Biol. Chem.*, 265, 16455-16463, 1990), anti-insulin-like growth factor antibody (Yao et al., *J. Neurosci. Res.*, 40, 647-659, 1995), anti-insulin-like growth factor receptor antibody (Yao et al., *J. Neurosci. Res.*, 40, 647-659, 1995), anti-PMSA antibody (Murphy et al., *J. Urology*, 160, 2396-2401, 1998), anti-vascular endothelial cell growth factor antibody (Presta et al., *Cancer Res.*, 57, 4593-4599, 1997), anti-vascular endothelial cell growth factor receptor antibody (Kanno et al., *Oncogene*, 19, 2138-2146, 2000) and the like. Examples of the antibody which recognizes an allergy- or inflammation-related antigen include anti-interleukin 6 antibody (Abrams et al., *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 6 receptor antibody (Sato et al., *Molecular Immunol.*, 31, 371-381, 1994), anti-interleukin 5 antibody (Abrams et al., *Immunol. Rev.*, 127, 5-24, 1992), anti-interleukin 5 receptor antibody and anti-interleukin 4 antibody (Biord et al., *Cytokine*, 3, 562-567, 1991), anti-tumor necrosis factor antibody (Tempest et al., *Hybridoma*, 13, 183-190, 1994), anti-tumor necrosis factor receptor antibody (Amrani et al., *Molecular Pharmacol.*, 58, 237-245, 2000), anti-CCR4 antibody (Campbell et al., *Nature*, 400, 776-780, 1999), anti-chemokine antibody (Peri et al., *J. Immuno. Meth.*, 174, 249-257, 1994), anti-chemokine receptor antibody (Wu et al., *J. Exp. Med.*, 186, 1373-1381, 1997) and the like. Examples of the antibody which recognizes a circulatory organ disease-related antigen include anti-GpIIb/IIIa antibody (Co et al., *J. Immunol.*, 152, 2968-2976, 1994), anti-platelet-derived growth factor antibody (Ferns et al., *Science*, 253, 1129-1132, 1991), anti-platelet-derived growth factor receptor antibody (Shulman et al., *J. Biol. Chem.*, 272, 17400-17404, 1997) and anti-blood coagulation factor antibody (Peter et al., *Circulation*, 101, 1158-1164, 2000) and the like. Examples of the antibody

which recognizes a viral or bacterial infection-related antigen include anti-gp120 antibody (Tugarinov et al., *Structure*, **8**, 385-395, 2000), anti-CD4 antibody (Schulze-Koops et al., *J. Rheumatology*, **25**, 2065-2076, 1998), anti-CCR4 antibody and anti-Vero toxin antibody (Karnali et al., *J. Clin. Microbiol.*, **37**, 396-399, 1999) and the like.

These antibodies can be obtained from public organizations such as ATCC (The American Type Culture Collection), RIKEN Gene Bank at The Institute of Physical and Chemical Research, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology and the like, or private reagent sales companies such as Dainippon Pharmaceutical, R & D SYSTEMS, PharMingen, Cosmo Bio, Funakoshi and the like.

(0066)

In the present invention, the effector function of the immunologically functional molecule means cytotoxic activity through Fc region (ADCC activity) and/or complement-dependent cytotoxic activity (CDC activity).

The cytotoxic activity through Fc region is an activity in which an antibody bound to a cell surface antigen on a tumor cell in the living body activate an effector cell through an Fc receptor existing on the antibody Fc region and the surface of an effector cell such as a killer cell, a natural killer cell, an activated macrophage and the like and thereby obstruct the tumor cell and the like [*Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc., Chapter 2.1 (1955)].

(0067)

As the protein or peptide as the above immunologically functional molecule, any one can be used, so long as it can activate various immunological reactions. Examples include interferon molecules, such as interleukin-2 (IL-2) (*Science*, **193**, 1007 (1976)) and interleukin-12 (IL-12) (*J. Leuc. Biol.*, **55**, 280 (1994)); colony-

stimulating factors, such as granulocyte colony-stimulating factor (G-CSF) (*J. Biol. Chem.*, 258, 9017 (1983)), macrophage colony-stimulating factor (M-CSF) (*J. Exp. Med.*, 173, 269 (1992)) and granulocyte macrophage colony-stimulating factor (MG-CSF) (*J. Biol. Chem.*, 252, 1998 (1977)); growth factors, such as erythropoietin (EPO) (*J. Biol. Chem.*, 252, 5558 (1977)) and thrombopoietin (TPO) (*Nature*, 369, 533 (1994)); and the like.

(0068)

The activities of protein and peptide of the present invention are activities of various immunocompetent cells including lymphocytes (T cell, B cell and the like) and macrophage, or various immune response reactions, when the sugar chain-containing protein and peptide are administered into the living body.

The promotion of activities of protein and peptide of the present invention includes activation of NK cell and T cell by IL-2 and IL-12, promotion activities of erythrocyte production by EPO and the like which are further increased.

The host cell of the present invention may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like. Examples of the cells include those which will be later in the item 3. Among animal cells, preferred examples include a CHO cell derived from a Chinese hamster ovary tissue, a rat myeloma cell line YB2/3HL.P2.G11.16Ag.20 cell, a mouse myeloma cell line NSO cell, a mouse myeloma SP2/0-Ag14 cell, a BHK cell derived from a syrian hamster kidney tissue, an antibody producing-hybridoma cell, a human leukemia cell line Namalwa cell, an embryonic stem cell, a fertilized egg cell and the like.

(0069)

In the present invention, the synteny means a genome gene region preserved on chromosome between heterogeneous species. That is, it is known that during evolution in organisms, there is a region in which a gene sequence is preserved between different organisms in a mosaic form in the genome. The part is called synteny. The synteny can be exemplified by The Dysmorphic Human-Mouse Homology Database (GHMHD, <http://www.hgmp.mrc.ac.uk/DHMHD/dysmorph.html>) or National Center for Biotechnology Information (<http://www3.ncbi.nlm.nih.gov/Homology>). (0070)

In the present invention, a DNA which hybridizes under stringent conditions is a DNA obtained, e.g., by a method such as colony hybridization, plaque hybridization or Southern blot hybridization using a DNA such as the DNA having the nucleotide sequence represented by SEQ ID NO:1, 2 or 3 or a partial fragment thereof as the probe, and specifically includes a DNA which can be identified by carrying out hybridization at 65°C in the presence of 0.7 to 1.0 M sodium chloride using a filter to which colony- or plaque-derived DNA fragments are immobilized, and then washing the filter at 65°C using 0.1 to 2 × SSC solution (composition of the 1 × SSC solution comprising 150 mM sodium chloride and 15 mM sodium citrate). The hybridization can be carried out in accordance with the methods described, e.g., in *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as "*Molecular Cloning, Second Edition*"), *Current Protocols in Molecular Biology*, John Wiley & Sons, 1987-1997 (hereinafter referred to as "*Current Protocols in Molecular Biology*"); *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Edition, Oxford University (1995); and the like. Examples of the hybridizable DNA include a DNA having at least 60% or more,

preferably 70% or more, more preferably 80% or more, still more preferably 90% or more, far more preferably 95% or more, and most preferably 98% or more, of homology with the nucleotide sequence represented by SEQ ID NO:1, 2 or 3.

Explanation of the target vector comprising the DNA is in accordance with the explanation described in the 1(4)(b) described below.

(0071)

The present invention will be described below in detail.

#### 1. Preparation of cell of the present invention

The cell in which the activity of an enzyme relating to the modification of a sugar chain of a sugar protein is controlled by an artificial technique of the present invention can be prepared by (a) adding an inhibitor or activator for the activity of the enzyme to a medium; (b) selecting a mutant relating to the enzyme; (c) introducing a gene encoding the enzyme; (d) a gene disruption for targeting a gene encoding the enzyme; (e) inhibiting transcription and/or translation of a gene encoding the enzyme. They are specifically explained below.

(0072)

(1) Preparation of cell of the present invention by addition of enzyme inhibitor or activator to medium

The cell of the present invention can be produced by adding an inhibitor or activator of the activity of an enzyme relating to the modification of a sugar strain of a glycoprotein.

The host cell of the present invention can be produced by culturing the cell in a medium to which an inhibitor or activator of the activity of an enzyme relating to the modification of a sugar strain of a glycoprotein has been added.

The enzyme inhibitor or activator to be added for the preparation of the host cell of the present invention

may be any compound, so long as it can change the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein. Examples of the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein include inhibitors of enzyme activity such as tunicamycin, caspato spermine, N-methyl-1-deoxynojirimycin, bromoconduritol, 1-deoxynojirimycin, 1,4-dioxy-1,4-imino-D-mannitol, swansonin, 1-deoxynojirimycin and the like. Also, it is known that deoxy derivatives of a substrate of an enzyme relating to the modification of a sugar chain influence the activity of an enzyme, and are exemplified as the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a sugar protein. Moreover, the compound which can change the activity of an enzyme relating to the modification of a sugar chain of a sugar protein includes a transcription activator or inhibitor which control the transcription of an enzyme gene relating to the modification of a sugar chain of a glycoprotein. The inhibitors or activators of the enzyme relating to the modification of a sugar chain of a glycoprotein may be added to a medium alone or to a medium culturing the host cell as a combination of plural compounds.

(0073)

(2) Preparation of cell of the present invention by selection of mutant

The method for selecting a desired cell line from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained includes a method in which a desired cell line is selected from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained as a measure of the activity of an enzyme relating to the modification of a sugar protein of a

glycoprotein, a method in which a desired cell line is selected from mutants obtained by treating the parent strain by a mutation-inducing treatment or mutants spontaneously obtained as a measure of the sugar chain structure of a glycoprotein produced from the mutants obtained by treating the parent strain by a mutation-inducing treatment or the mutants spontaneously obtained.

As the mutation-inducing treatment, any treatment can be used, so long as it can induce a point mutation or a deletion or frame shift mutation in the DNA of cells of the parent cell line. Examples include treatment with ethyl nitrosourea, nitrosoguanidine, benzopyrene or an acridine pigment and treatment with radiation. Also, various alkylating agents and carcinogens can be used as mutagens. Examples of the method for allowing a mutagen to act upon cells include the methods described in *Tissue Culture Techniques*, 3rd edition (Asakura Shoten), edited by Japanese Tissue Culture Association (1996), *Nature Genet.*, 24, 314 (2000) and the like.

Examples of the spontaneously generated mutant include mutants which are spontaneously formed by continuing subculture under general cell culture conditions without applying special mutation-inducing treatment.

(0074)

A method for selecting and measuring the activity of an enzyme relating to the modification of a glycoprotein include biochemical method, genetic method and the like described in the 7 below. Examples of the method for identifying the sugar chain structure of a glycoprotein include a method using lectin described in *Somatic Cell Mol. Genet.*, 12, 51 (1986) and the like. Examples of the lectin include WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (concanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil

agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (*Aleuria aurantia* lectin), ACL (*Amaranthus caudatus* lectin), BPL (*Bauhinia purpurea* lectin), DSL (*Datura stramonium* lectin), DBA (*Dolichos biflorus* agglutinin), EBL (elderberry bark lectin), ECL (*Erythrina cristagalli* lectin), EEL (*Euonymus europaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HHL (*Hippeastrum hybrid* lectin), Jacalin, LTL (*Lotus tetragonolobus* lectin), LEL (*Lycopersicon esculentum* lectin), MAL (*Maackia amurensis* lectin), MPL (*Maclura pomifera* lectin), NPL (*Narcissus pseudonarcissus* lectin), PNA (peanut agglutinin), E-PHA (*Phaseolus vulgaris* erythroagglutinin), PTL (*Psophocarpus tetragonolobus* lectin), RCA (*Ricinus communis* agglutinin), STL (*Solanum tuberosum* lectin), SJA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

(0075)

(3) Preparation of cell of the present invention by introduction of gene encoding enzyme relating to modification of sugar chain of glycoprotein

The cell of the present invention can be prepared, for example, as follows using the gene introduction method described in *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology*, *Manipulating the Mouse Embryo*, 2nd ed. and the like.

(0076)

cDNA of an enzyme relating to the modification of a sugar chain of a sugar protein is prepared.

If necessary, using the prepared full cDNA of an enzyme relating to the modification of a sugar chain of a sugar protein, a DNA fragment having an appropriate length comprising a part encoding the protein.

The DNA fragment or the full length cDNA is inserted into downstream of a promoter of an appropriate expression vector.

The recombinant vector is introduced to a host cell suitable for the expression vector to obtain a transformant.

The transformant is selected as a measure of the activity of an enzyme relating to the modification of the glycoprotein or the sugar chain structure of the glycoprotein.

The host cell may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like, such as the host cell described in the item 1.

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the desired enzyme relating to the modification of a sugar chain of a glycoprotein can be transferred is used. Examples include expression vectors which will be described later in the item 3.

(0077)

As the introduction of a gene into various host cells, the introduction method of a recombinant vector suitable for various host cells described later in the item 3 is used.

The method for selecting the transformant as a measure of the activity of the introduced enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein includes the methods described in the item 1(2).

cDNA of the enzyme relating to the modification of a sugar chain of a glycoprotein can be prepared, for example, as follows from human or non-human tissue or cell.

A total RNA or mRNA is prepared from a human or non-human animal tissue or cell.

A cDNA library is prepared from the prepared total RNA or mRNA.

(0078)

Degenerative primers are produced based on the amino acid sequence of an enzyme relating to the modification of a sugar chain of a glycoprotein, and a gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein is obtained by PCR using the prepared cDNA library as the template.

A DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein can be obtained by screening the cDNA library using the obtained gene fragment as a probe.

(0078)

Regarding the mRNA of a human or non-human tissue or cell, a commercially available product (e.g., manufactured by Clontech) may be used or it may be prepared from a human or non-human animal tissue or cell in the following manner. Examples of the method for preparing a total RNA from a human or non-human animal tissue or cell include the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)], the acidic guanidine thiocyanate phenol chloroform (AGPC) method [*Analytical Biochemistry*, 162, 156 (1987); *Experimental Medicine*, 2, 1937 (1991)] and the like.

(0080)

Also, examples of the method for preparing mRNA from a total RNA as poly(A)<sup>+</sup> RNA include an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like.

In addition, mRNA can be prepared using a kit such as Fast Track mRNA Isolation Kit (manufactured by

Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) or the like.

A cDNA library is prepared from the prepared mRNA of a human or non-human animal tissue or cell. Examples of the method for preparing cDNA libraries include the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology; A Laboratory Manual*, Second Edition (1989); and the like, or methods using commercially available kits such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies), ZAP-cDNA Synthesis Kit (manufactured by STRATAGENE) and the like.

(0081)

As the cloning vector for use in the preparation of the cDNA library, any vector such as a phage vector, a plasmid vector or the like can be used, so long as it is autonomously replicable in *Escherichia coli* K12. Examples include ZAP Express [manufactured by STRATAGENE, *Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)], Lambda ZAP II (manufactured by STRATAGENE),  $\lambda$ gt10 and  $\lambda$ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)],  $\lambda$ TriplEx (manufactured by Clontech),  $\lambda$ ExCell (manufactured by Pharmacia), pCD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

(0082)

Any microorganism can be used as the host microorganism, but *Escherichia coli* is preferably used. Examples include *Escherichia coli* XL1-Blue MRF' [manufactured by STRATAGENE, *Strategies*, 5, 81 (1992)], *Escherichia coli* C600 [*Genetics*, 39, 440 (1954)], *Escherichia coli* Y1088 [*Science*, 222, 778 (1983)], *Escherichia coli* Y1090 [*Science*, 222, 778 (1983)], *Escherichia coli* NM522 [*J. Mol. Biol.*, 166, 1 (1983)],

*Escherichia coli* K802 [*J. Mol. Biol.*, 16, 118 (1966)], *Escherichia coli* JM105 [*Gene*, 38, 275 (1985)] and the like (0083)

The cDNA library may be used as such in the succeeding analysis, and in order to obtain a full length cDNA as efficient as possible by decreasing the ratio of an infull length cDNA, a cDNA library prepared using the oligo cap method developed by Sugano et al. [*Gene*, 138, 171 (1994); *Gene*, 200, 149 (1997); *Protein, Nucleic Acid and Protein*, 41, 603 (1996); *Experimental Medicine*, 11, 2491 (1993); *cDNA Cloning* (Yodo-sha) (1996); *Methods for Preparing Gene Libraries* (Yodo-sha) (1994)] may be used in the following analysis.

(0084)

Degenerative primers specific for the 5'-terminal and 3'-terminal nucleotide sequences of a nucleotide sequence presumed to encode the amino acid sequence are prepared based on the amino acid sequence of the enzyme relating to modification of a sugar chain of a glycoprotein, and DNA is amplified by PCR [*PCR Protocols*, Academic Press (1990)] using the prepared cDNA library as the template to obtain a gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein.

(0085)

It can be confirmed that the obtained gene fragment is a DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein, by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems) or the like.

A DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can be obtained by carrying out colony hybridization or plaque hybridization (*Molecular Cloning*, Second Edition) for the cDNA or cDNA

library synthesized from the mRNA contained in the human or non-human animal tissue or cell, using the gene fragment as a DNA probe.

(0086)

Also, a DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be obtained by carrying out screening by PCR using the cDNA or cDNA library synthesized from the mRNA contained in a human or non-human animal tissue or cell as the template and using the primers used for obtaining the gene fragment encoding the enzyme relating to modification of a sugar chain of a glycoprotein.

The nucleotide sequence of the obtained DNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein is analyzed from its terminus and determined by a method usually used for analyzing a nucleotide, such as the dideoxy method of Sanger et al. [*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)], a nucleotide sequence analyzer such as ABIPRISM 377 DNA Sequencer (manufactured by PE Biosystems) or the like.

(0087)

A gene encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be determined from genes in data bases by searching nucleotide sequence data bases such as GenBank, EMBL, DDBJ and the like using a homology retrieving program such as BLAST based on the determined cDNA nucleotide sequence.

Examples of the nucleotide sequence of the gene obtained by the method encoding the enzyme relating to modification of a sugar chain of a glycoprotein include the nucleotide sequence represented by SEQ ID NO:1 or 2.

The cDNA encoding the enzyme relating to modification of a sugar chain of a glycoprotein can also be obtained by chemically synthesizing it with a DNA synthesizer such as DNA Synthesizer model 392 manufactured

by Perkin Elmer or the like using the phosphoamidite method, based on the determined DNA nucleotide sequence.

(0088)

(4) Preparation of cell of the present invention by disruption of gene encoding enzyme relating to modification of sugar chain of sugar protein

The host cell of the present invention can be prepared using a gene disruption technique by targeting an enzyme relating to the modification of a sugar chain of a glycoprotein. The gene as used herein includes DNA and RNA.

The gene disruption method may be any method, so long as it can disrupt the gene of the target enzyme relating to the modification of a sugar chain of a glycoprotein is included. Examples include an antisense method, a ribozyme method, a homologous recombination method, an RDO method, an RNAi method, a retrovirus-employed method, a transposon-employed method and the like. The methods are specifically described below.

(a) Preparation of the host cell of the present invention by the antisense method or the ribozyme method

The host cell of the present invention can be prepared by the ribozyme method described in *Cell Technology*, 12, 239 (1993); *BIO/TECHNOLOGY*, 17, 1097 (1999); *Hum. Mol. Genet.*, 5, 1083 (1995); *Cell Technology*, 13, 255 (1994); *Proc. Natl. Acad. Sci. USA*, 96, 1886 (1999); or the like, e.g., in the following manner by targeting at an enzyme relating to the modification of a sugar chain of a glycoprotein.

(0089)

A cDNA or a genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

Based on the determined DNA sequence, an appropriate length of an antisense gene or ribozyme construct comprising a DNA moiety which encodes the enzyme relating to the modification of a sugar chain of a glycoprotein, a part of its non-translation region or an intron, is designed.

In order to express the antisense gene or ribozyme in a cell, a recombinant vector is prepared by inserting a fragment or total length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

The host cell of the present invention can be obtained by selecting a transformant using, as a marker, the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein. The host cell of the present invention can also be obtained by selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane or the sugar chain structure of the produced antibody molecule.

As the host cell used for the production of the host cell of the present invention, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include host cells which will be described later in the item 3.

(0090)

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed antisense gene or ribozyme can be transferred is used. Examples include

expression vectors which will be described later in the item 3.

Regarding the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

As the method for selecting a transformant as a measure of the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein, the method described in the item 1(2) can be exemplified.

(0091)

A method for preparing cDNA of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, includes the method described in the item 1(3).

A method for preparing a genome DNA of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, includes the method described in the item 1(4)(b).

Furthermore, the host cell of the present invention can be obtained by directly introducing an antisense oligonucleotide or ribozyme decided based on the nucleotide sequence of an enzyme relating to the modification of a sugar chain of a glycoprotein into a host cell.

(0092)

The antisense oligonucleotide or ribozyme can be prepared in the usual method or using a DNA synthesizer. Specifically, it can be prepared based on the sequence information of an oligonucleotide having a corresponding sequence of continued 5 to 150 bases, preferably 5 to 60 bases, and more preferably 10 to 40 bases, among nucleotide sequences of a cDNA and a genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein, by synthesizing an oligonucleotide which corresponds to a sequence complementary to the

oligonucleotide (antisense oligonucleotide) or a ribozyme comprising the oligonucleotide sequence.

(0093)

Examples of the oligonucleotide include oligo RNA and derivatives of the oligonucleotide (hereinafter referred to as "oligonucleotide derivatives").

Examples of the oligonucleotide derivatives include oligonucleotide derivatives in which a phosphodiester bond in the oligonucleotide is converted into a phosphorothioate bond, an oligonucleotide derivative in which a phosphodiester bond in the oligonucleotide is converted into an N3'-P5' phosphoamidate bond, an oligonucleotide derivative in which ribose and a phosphodiester bond in the oligonucleotide are converted into a peptide-nucleic acid bond, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 propynyluracil, an oligonucleotide derivative in which uracil in the oligonucleotide is substituted with C-5 thiazoleuracil, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with C-5 propynylcytosine, an oligonucleotide derivative in which cytosine in the oligonucleotide is substituted with phenoxazine-modified cytosine, an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-O-propylribose and an oligonucleotide derivative in which ribose in the oligonucleotide is substituted with 2'-methoxyethoxyribose [*Cell Technology*, 16, 1463 (1997)].

(0094)

(b) Preparation of cell of the present invention by homologous recombination

The cell of the present invention can be produced by modifying a target gene on chromosome through a homologous recombination technique, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein as the target gene.

The target gene on the chromosome can be modified by using a method described in *Manipulating the Mouse Embryo, A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1994) (hereinafter referred to as "*Manipulating the Mouse Embryo, A Laboratory Manual*"); *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995) (hereinafter referred to as "*Preparation of Mutant Mice using ES Cells*"); or the like, for example, as follows.

(0095)

A genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

Based on the nucleotide sequence of the genome DNA, a target vector is prepared for homologous recombination of a target gene to be modified (e.g., structural gene of the enzyme relating to the modification of a sugar chain of a glycoprotein, or a promoter gene).

The host cell of the present invention can be produced by introducing the prepared target vector into a host cell and selecting a cell in which homologous recombination occurred between the target gene and target vector.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3.

(0096)

Examples of the method for preparing genome DNA include known methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; and the like. In addition, a genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein can also be isolated using a kit such as

Genome DNA Library Screening System (manufactured by Genome Systems), Universal GenomeWalker™ Kits (manufactured by CLONTECH) or the like.

Examples of the nucleotide sequence of genome DNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the nucleotide sequence represented by SEQ ID NO:3.

The target vector for use in the homologous recombination of the target gene can be prepared in accordance with a method described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. The target vector can be used as either a replacement type or an insertion type.

(0097)

For introducing the target vector into various host cells, the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3, can be used.

Examples of the method for efficiently selecting a homologous recombinant include a method such as the positive selection, promoter selection, negative selection or polyA selection described in *Gene Targeting, A Practical Approach*, IRL Press at Oxford University Press (1993); *Biomanual Series 8, Gene Targeting, Preparation of Mutant Mice using ES Cells*, Yodo-sha (1995); or the like. Examples of the method for selecting the homologous recombinant of interest from the selected cell lines include the Southern hybridization method for genome DNA (*Molecular Cloning*, Second Edition), PCR [*PCR Protocols*, Academic Press (1990)], and the like.

(0098)

(c) Preparation of cell of the present invention by RDO method

The cell of the present invention can be prepared by an RDO (RNA-DNA oligonucleotide) method by targeting at a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, as follows.

A cDNA or a genome DNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA or genome DNA is determined.

Based on the determined DNA sequence, an appropriate length of an RDO construct comprising a DNA moiety which encodes the enzyme relating to the modification of a sugar chain of a glycoprotein or a part of its non-translation region or an intron, is designed and synthesized.

The host cell of the present invention can be obtained by introducing the synthesized RDO into a host cell and then selecting a transformant in which a mutation occurred in the target enzyme relating to the modification of a sugar chain of a glycoprotein.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target. Examples include the host cells which will be described later in the item 3.

Examples of the method for introducing RDO into various host cells include the methods for introducing recombinant vectors suited for various host cells, which will be described later in the item 3.

Examples of the method for preparing cDNA encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the methods described in the preparation of DNA in the item 1(3) and the like.

(0099)

Examples of the method for preparing a genome DNA encoding the enzyme relating to the modification of a sugar

chain of a glycoprotein include the methods in preparation of genome DNA described in the item 1(4)(a) and the like.

The method for determining the nucleotide sequence of DNA includes the method described in item 6.

The RDO can be prepared by a usual method or using a DNA synthesizer.

Examples of the method for selecting a cell in which a mutation occurred, by introducing the ROD into the host cell, in the gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein include the methods for directly detecting mutations in chromosomal genes described in *Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology* and the like. Also, the method of the item 1(2) in which the transformant can be selected as a measure of the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein can be used.

(0100)

The construct of the ROD can be designed in accordance with the methods described in *Science*, 273, 1386 (1996); *Nature Medicine*, 4, 285 (1998); *Hepatology*, 25, 1462 (1997); *Gene Therapy*, 5, 1960 (1999); *J. Mol. Med.*, 75, 829 (1997); *Proc. Natl. Acad. Sci. USA*, 96, 8774 (1999); *Proc. Natl. Acad. Sci. USA*, 96, 8768 (1999); *Nuc. Acids. Res.*, 27, 1323 (1999); *Invest. Dermatol.*, 111, 1172 (1998); *Nature Biotech.*, 16, 1343 (1998); *Nature Biotech.*, 18, 43 (2000); *Nature Biotech.*, 18, 555 (2000); and the like.

(0101)

(d) Preparation of cell of the present invention by RNAi method

The cell of the present invention can be prepared by the RNAi (RNA interference) method by targeting at a gene of an enzyme relating to the modification of a sugar chain of a glycoprotein, for example, as follows.

A cDNA encoding an enzyme relating to the modification of a sugar chain of a glycoprotein is prepared.

The nucleotide sequence of the prepared cDNA is determined.

Based on the determined DNA sequence, an appropriate length of an RNAi gene construct comprising the DNA coding moiety encoding the enzyme relating to the modification of a sugar chain of a glycoprotein or a part of its non-translation region, is designed.

In order to express the RNAi gene in a cell, a recombinant vector is prepared by inserting a fragment or full length of the prepared DNA into downstream of the promoter of an appropriate expression vector.

A transformant is obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

The host cell of the present invention can be obtained by selecting a transformant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein, or the sugar chain structure of a glycoprotein.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3.

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the designed RNAi gene can be transferred is used. Examples include the expression vectors which will be described later in the item 3.

(0102)

As the method for introducing a gene into various host cells, the methods for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a transformant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein include the methods described in the item 1(2).

Examples of the method for selecting a transformant as a measure of the sugar chain structure of a glycoprotein on the cell membrane include the methods which will be described later in the item 1(3).

In addition, the host cell of the present invention can also be obtained without using an expression vector, by directly introducing an RNAi gene designed based on the nucleotide sequence encoding the enzyme relating to the modification of a sugar chain of a glycoprotein.

The RNAi gene can be prepared in the usual method or using a DNA synthesizer.

(0103)

The RNAi gene construct can be designed in accordance with the methods described in *Nature*, 391, 806 (1998); *Proc. Natl. Acad. Sci. USA*, 95, 15502 (1998); *Nature*, 395, 854 (1998); *Proc. Natl. Acad. Sci. USA*, 96, 5049 (1999); *Cell*, 95, 1017 (1998); *Proc. Natl. Acad. Sci. USA*, 96, 1451 (1999); *Proc. Natl. Acad. Sci. USA*, 95, 13959 (1998); *Nature Cell Biol.*, 2, 70 (2000); and the like.

(0104)

(e) Preparation of cell of the present invention by method using transposon

The cell of the present invention can be prepared by inducing mutation using a transposon system described in *Nature Genet.*, 25, 35 (2000) or the like, and then by selecting a mutant as a measure of the activity of the

enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein.

The transposon system is a system in which a mutation is induced by randomly inserting an exogenous gene into chromosome, wherein an exogenous gene interposed between transposons is generally used as a vector for inducing a mutation, and a transposase expression vector for randomly inserting the gene into chromosome is introduced into the cell at the same time.

(0105)

Any transposase can be used, so long as it is suitable for the sequence of the transposon to be used.

As the exogenous gene, any gene can be used, so long as it can induce a mutation in the DNA of a host cell.

As the host cell, any cell such as yeast, animal cell, insect cell or plant cell can be used, so long as it has a gene encoding the target enzyme relating to the modification of a sugar chain of a glycoprotein. Examples include the host cells which will be described later in the item 3. For introducing the gene into various host cells, the method for introducing recombinant vectors suitable for various host cells, which will be described later in the item 3, can be used.

Examples of the method for selecting a mutant as a measure of the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein or the sugar chain structure of a glycoprotein include the methods described in the item 1(2).

(0106)

(5) Method for inhibiting transcription and/or translation of gene encoding enzyme relating to modification of sugar chain of glycoprotein

The cell of the present invention can be prepared by inhibiting transcription and/or translation of a target gene through a method such as the antisense RNA/DNA

technique [*Bioscience and Industry*, 50, 322 (1992); *Chemistry*, 46, 681 (1991); *Biotechnology*, 9, 358 (1992); *Trends in Biotechnology*, 10, 87 (1992); *Trends in Biotechnology*, 10, 152 (1992); *Cell Engineering*, 16, 1463 (1997)], the triple helix technique [*Trends in Biotechnology*, 10, 132 (1992)] or the like, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, as the target.

(0107)

2. Preparation of a transgenic non-human animal or plant or the progenies thereof of the present invention

The transgenic non-human animal or plant or the progenies thereof of the present invention is a transgenic non-human animal or plant or the progenies thereof in which a genome gene is modified in such a manner that the activity of an enzyme relating to the modification of a sugar chain of an antibody molecule can be controlled, and it can be prepared according to the method similar to that in the item 1, using a gene encoding an enzyme relating to the modification of a sugar chain of a glycoprotein, as the target.

(0108)

In a transgenic non-human animal, the embryonic stem cell of the present invention in which the activity of the enzyme the modification of a sugar chain of a glycoprotein is controlled can be prepared applying the method similar to that in the item 1 to an embryonic stem cell of the intended non-human animal such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit or the like. Specifically, a mutant clone is prepared in which a gene encoding the enzyme relating to the modification of a sugar chain of a glycoprotein is inactivated or substituted with any sequence, by a known homologous recombination technique [e.g., *Nature*, 326, 6110, 295 (1987); *Cell*, 51, 3, 503 (1987); or the like]. Using the prepared mutant clone,

a chimeric individual comprising an embryonic stem cell clone and a normal cell can be prepared by an injection chimera method into blastocyst of fertilized egg of an animal or by an aggregation chimera method. The chimeric individual is crossed with a normal individual, so that a transgenic non-human animal in which the activity of the enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted in the whole body cells can be obtained.

(0109)

Also, a fertilized egg cell of the present invention in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted can be prepared by applying the method similar to that in the item 1 to fertilized egg of a non-human animal of interest such as cattle, sheep, goat, pig, horse, mouse, rat, fowl, monkey, rabbit or the like. A transgenic non-human animal in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased can be prepared by transplanting the prepared fertilized egg cell into the oviduct or uterus of a pseudopregnant female using the embryo transplantation method described in *Manipulating Mouse Embryo*, Second Edition or the like, followed by childbirth by the animal.

(0110)

In a transgenic plant, the callus of the present invention in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased or deleted can be prepared by applying the method similar to that in the item 1 to a callus or cell of the plant of interest. A transgenic plant in which the activity of an enzyme relating to the modification of a sugar chain of a glycoprotein is decreased can be prepared by culturing the prepared callus using a medium comprising auxin and cytokinin to redifferentiate it in accordance with

a known method [*Tissue Culture*, 20 (1994); *Tissue Culture*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)].

(0111)

3. Method for producing immunologically functional molecule expressed in various cells

The immunologically functional molecule can be obtained by expressing it in a host cell using the methods described in *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*; *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988 (hereinafter referred also to as "Antibodies"); *Monoclonal Antibodies: Principles and Practice*, Third Edition, Acad. Press, 1993 (hereinafter referred also to as "Monoclonal Antibodies"); and *Antibody Engineering, A Practical Approach*, IRL Press at Oxford University Press (hereinafter referred also to as "Antibody Engineering"), for example, as follows.

(0102)

A full length cDNA encoding an immunologically functional molecule is prepared, and an appropriate length of a DNA fragment comprising a moiety encoding the antibody molecule is prepared.

A recombinant vector is prepared by inserting the DNA fragment or the full length cDNA into downstream of the promoter of an appropriate expression vector.

A transformant which produces the antibody molecule can be obtained by introducing the recombinant vector into a host cell suitable for the expression vector.

As the host cell, any of yeast, animal cell, insect cell, plant cell or the like can be used, so long as it can express the gene of interest.

A cell such as yeast, animal cell, insect cell, plant cell or the like into which an enzyme relating to the modification of an N-glycoside-linked sugar chain which binds to the Fc region of the antibody molecule is

introduced by a genetic engineering technique can also be used as the host cell.

(0103)

As the expression vector, a vector which is autonomously replicable in the host cell or can be integrated into the chromosome and comprises a promoter at such a position that the DNA encoding the immunologically functional molecule of interest can be transferred is used.

The cDNA can be prepared from a human or non-human tissue or cell using, e.g., a probe primer specific for the immunologically functional molecule of interest, in accordance with the methods described in the preparation of DNA in the item 1(1)(a).

(0114)

When a procaryote cell, such as a bacterium or the like, is used as the host cell, it is preferred that the recombinant vector containing the DNA encoding the polypeptide of the present invention can replicate autonomously in the bacterium and is a recombinant vector constituted by, at least a promoter, a ribosome binding sequence, the DNA of the present invention and a transcription termination sequence. A promoter controlling gene can also be contained therewith in operable combination.

(0115)

Examples of the expression vectors include a vector plasmid which is replicable in *Corynebacterium glutamicum*, such as pCG1 (Japanese Published Unexamined Patent Application No. 134500/82), pCG2 (Japanese Published Unexamined Patent Application No. 35197/83), pCG4 (Japanese Published Unexamined Patent Application No. 183799/82), pCG11 (Japanese Published Unexamined Patent Application No. 134500/82), pCG116, pCE54 and pCB101 (Japanese Published Unexamined Patent Application No. 105999/83), pCE51, pCE52 and pCE53 (*Mol. Gen. Genet.*, 196: 175-178 (1984)), and the

like; a vector plasmid which is replicable in *Escherichia coli*, such as pET3 and pET11 (manufactured by Stratagene), pBAD, pThioHis and pTrcHis (manufactured by Invitrogen), pKK223-3 and pGEX2T (manufactured by Amersham Pharmacia Biotech), and the like; and pBTrp2, pBTac1 and pBTac2 (manufactured by Boehringer Mannheim Co.), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 (*Agric. Biol. Chem.*, 48: 669 (1984)), pLSA1 (*Agric. Biol. Chem.*, 53: 277 (1989)), pGEL1 (*Proc. Natl. Acad. Sci. USA*, 82: 4306 (1985)), pBluescript II SK(-) (manufactured by Stratagene), pTrs30 (prepared from *Escherichia coli* JM109/pTrs30 (FERM BP-5407)), pTrs32 (prepared from *Escherichia coli* JM109/pTrs32 (FERM BP-5408)), pGHA2 (prepared from *Escherichia coli* IGHA2 (FERM B-400), Japanese Published Unexamined Patent Application No. 221091/85), pGKA2 (prepared from *Escherichia coli* IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No. 221091/85), pTerm2 (U.S. Patents 4,686,191, 4,939,094 and 5,160,735), pSupex, pUB110, pTP5, pC194 and pEG400 (*J. Bacteriol.*, 172: 2392 (1990)), pGEX (manufactured by Pharmacia), pET system (manufactured by Novagen), and the like.

Any promoter can be used so long as it can function in the host cell. Examples include promoters derived from *Escherichia coli*, phage and the like, such as trp promoter ( $P_{trp}$ ), lac promoter,  $P_L$  promoter,  $P_R$  promoter, T7 promoter and the like. Also, artificially designed and modified promoters, such as a promoter in which two  $P_{trp}$  are linked in series ( $P_{trp} \times 2$ ), tac promoter, lacT7 promoter letI promoter and the like, can be used.

(0116)

It is preferred to use a plasmid in which the space between Shine-Dalgarno sequence which is the ribosome

binding sequence and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides).

The transcription termination sequence is not always necessary for the expression of the DNA of the present invention. However, it is preferred to arrange the transcription terminating sequence at just downstream of the structural gene.

(0117)

Examples of the host cell include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Microbacterium*, the genus *Pseudomonas*, and the like. Specific examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* GI698, *Escherichia coli* TB1, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Corynebacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium glutamicum* ATCC 14067 (prior genus and species: *Brevibacterium flavum*), *Corynebacterium glutamicum* ATCC 13869 (prior genus and species: *Brevibacterium lactofermentum*, or *Corynebacterium lactofermentum*), *Corynebacterium acetoacidophilum* ATCC 13870, *Corynebacterium thermoaminogenes* FERM 9244, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas putida*, *Pseudomonas* sp. D-0110, and the like.

(0118)

With regard to the method for the introduction of the recombinant vector, any method for introducing DNA into

the above-described host cells, such as a method in which a calcium ion is used (*Proc. Natl. Acad. Sci. USA*, 69: 2110 (1972)), a protoplast method (Japanese Published Unexamined Patent Application No. 2483942/88), the methods described in *Gene*, 17: 107 (1982) and *Molecular & General Genetics*, 168: 111 (1979) and the like, can be used.

(0119)

When a yeast is used as the host cell, examples of the expression vector include YEP13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419) and the like.

Any promoter can be used, so long as it can function in yeast. Examples include a promoter of a gene of the glycolytic pathway such as a hexose kinase gene, etc., PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF  $\alpha$ 1 promoter, CUP 1 promoter and the like.

Examples of the host cell include microorganisms belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces* and the like, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*, etc.

(0120)

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into yeast. Examples include electroporation [*Methods in Enzymology*, 194, 182 (1990)], spheroplast method [*Proc. Natl. Acad. Sci. USA*, 84, 1929 (1978)], lithium acetate method [*J. Bacteriol.*, 153, 163 (1983)], a method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978) and the like.

(0121)

When an animal cell is used as the host, examples of the expression vector include pcDNAI, pcDM8 (available

from Funakoshi), pAGE107 [Japanese Published Examined Patent Application No. 22979/91; *Cytotechnology*, 3, 133 (1990)], pAS3-3 (Japanese Published Examined Patent Application No. 227075/90), pCDM8 [*Nature*, 329, 840 (1987)], pCDNAI/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [*J. Biochemistry*, 101, 1307 (1987)], pAGE210 and the like.

Any promoter can be used, so long as it can function in an animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), an early promoter of SV40, a promoter of retrovirus, a promoter of metallothionein, a heat shock promoter, an SR $\alpha$  promoter and the like. Also, an enhancer of the IE gene of human CMV may be used together with the promoter.

(0122)

Examples of the host cell include a human cell such as Namalwa cell, a monkey cell such as COS cell, a Chinese hamster cell such as CHO cell or HBT5637 (Japanese Published Examined Patent Application No. 299/88), a rat myeloma cell, a mouse myeloma cell, a cell derived from syrian hamster kidney, an embryonic stem cell, a fertilized egg cell and the like.

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into an animal cell. Examples include electroporation [*Cytotechnology*, 3, 133 (1990)], the calcium phosphate method (Japanese Published Examined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)], the injection method [*Manipulating the Mouse Embryo, A Laboratory Manual*], a method using particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813), the DEAE-dextran method [*Biomanual Series 4-Gene Transfer and Expression Analysis* (Yodo-sha), edited by Takashi Yokota and Kenichi Arai

(1994)], the virus vector method [*Manipulating Mouse Embryo*, Second Edition] and the like.

(0123)

When an insect cell is used as the host, the protein can be expressed by the method described in *Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992), *Bio/Technology*, 6, 47 (1988) or the like.

That is, the protein can be expressed by simultaneously introducing a recombinant gene-introducing vector and a baculovirus into an insect cell to obtain a recombinant virus in an insect cell culture supernatant and then infecting the insect cell with the recombinant virus.

Examples of the gene introducing vector used in the method include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus which is infected with an insect of the family *Barathra*.

(0124)

Examples of the insect cell include *Spodoptera frugiperda* oocytes Sf9 and Sf21 [*Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992)], a *Trichoplusia ni* oocyte High 5 (manufactured by Invitrogen) and the like.

Examples of the method for the simultaneously introducing the recombinant gene-introducing vector and the baculovirus for preparing the recombinant virus include the calcium phosphate method (Japanese Published Examined Patent Application No. 227075/90), the lipofection method [*Proc. Natl. Acad. Sci. USA*, 84, 7413 (1987)] and the like.

(0125)

When a plant cell is used as the host, examples of the expression vector include Ti plasmid, tobacco mosaic virus and the like.

As the promoter, any promoter can be used, so long as it can function in a plant cell. Examples include cauliflower mosaic virus (CaMV) 35S promoter, rice actin 1 promoter and the like.

Examples of the host cell include plant cells of tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, barley, etc., and the like.

(0126)

As the method for introducing the recombinant vector, any method can be used, so long as it can introduce DNA into a plant cell. Examples include a method using *Agrobacterium* (Japanese Published Examined Patent Application No. 140885/84, Japanese Published Examined Patent Application No. 70080/85, WO 94/00977), electroporation (Japanese Published Examined Patent Application No. 251887/85), a method using a particle gun (gene gun) (Japanese Patent No. 2606856, Japanese Patent No. 2517813) and the like.

As the method for expressing a gene, secretion production, expression of a fusion protein of the Fc region with other protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition or the like, in addition to the direct expression.

(0127)

When a gene is expressed by a bacterium, a yeast, an animal cell, an insect cell or a plant cell into which a gene relating to the synthesis of a sugar chain is introduced, an antibody molecule to which a sugar or a sugar chain is added by the introduced gene can be obtained.

(0128)

An immunologically functional molecule can be obtained by culturing the obtained transformant in a medium to produce and accumulate the antibody molecule in the culture and then recovering it from the resulting culture. The method for culturing the transformant using a medium can be carried out in accordance with a general method which is used for the culturing of host cells.

As the medium for culturing a transformant obtained using a prokaryote such as *Escherichia coli* etc. or a eukaryote such as yeast etc. as the host cell, the medium may be either a natural medium or a synthetic medium, so long as it comprises materials such as a carbon source, a nitrogen source, an inorganic salt and the like which can be assimilated by the organism and culturing of the transformant can be efficiently carried out.

(0129)

As the carbon source, those which can be assimilated by the organism can be used. Examples include carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch, starch hydrolysate, etc.; organic acids such as acetic acid, propionic acid, etc.; alcohols such as ethanol, propanol, etc.; and the like.

Examples of the nitrogen source include ammonia; ammonium salts of inorganic acid or organic acid such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, etc.; other nitrogen-containing compounds; peptone; meat extract; yeast extract; corn steep liquor; casein hydrolysate; soybean meal; soybean meal hydrolysate; various fermented cells and hydrolysates thereof; and the like.

Examples of the inorganic material include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, and the like

(0130)

The culturing is carried out generally under aerobic conditions such as a shaking culture, submerged-aeration stirring culture or the like. The culturing temperature is preferably 15 to 40°C, and the culturing time is generally 16 hours to 7 days. During the culturing, the pH is maintained at 3.0 to 9.0. The pH is adjusted using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

If necessary, an antibiotic such as ampicillin, tetracycline or the like may be added to the medium during the culturing.

When a microorganism transformed with a recombinant vector obtained using an inducible promoter as the promoter is cultured, an inducer may be added to the medium, if necessary. For example, when a microorganism transformed with a recombinant vector obtained using *lac* promoter is cultured, isopropyl- $\beta$ -D-thiogalactopyranoside may be added to the medium, and when a microorganism transformed with a recombinant vector obtained using *trp* promoter is cultured, indoleacrylic acid may be added to the medium.

(0131)

When a transformant obtained using an animal cell as the host cell is cultured, examples of the medium include generally used RPMI 1640 medium [*The Journal of the American Medical Association*, 199, 519 (1967)], Eagle's MEM medium [*Science*, 122, 501 (1952)], Dulbecco's modified MEM medium [*Virology*, 8, 396 (1959)], 199 medium [*Proceeding of the Society for the Biological Medicine*, 73, 1 (1950)] and Whitten's medium [*Developmental Engineering Experimentation Manual-Preparation of Transgenic Mice* (Kodan-sha), edited by M. Katshuki (1987)], the media to which fetal calf serum, etc. is added, and the like.

(0132)

The culturing is carried out generally at a pH of 6 to 8 and 30 to 40°C for 1 to 7 days in the presence of 5% CO<sub>2</sub>. If necessary, an antibiotic such as kanamycin, penicillin or the like may be added to the medium during the culturing.

Examples of the medium for use in the culturing of a transformant obtained using an insect cell as the host include usually used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM medium (manufactured by Life Technologies), ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium [*Nature*, 195, 788 (1962)] and the like.

The culturing is carried out generally at a medium pH of 6 to 7 and 25 to 30°C for 1 to 5 days.

In addition, antibiotics such as gentamicin may be added to the medium during the culturing as occasion demands.

(0133)

A transformant obtained using a plant cell as the host can be cultured as a cell or by differentiating it into a plant cell or organ. Examples of the medium for culturing the transformant include generally used Murashige and Skoog (MS) medium and White medium, the media to which a plant hormone such as auxin, cytokinin, etc. is added, and the like.

The culturing is carried out generally at a pH of 5 to 9 and 20 to 40°C for 3 to 60 days.

If necessary, an antibiotic such as kanamycin, hygromycin or the like may be added to the medium during the culturing.

(134)

Accordingly, an immunologically functional molecule can be produced by culturing a transformant derived from a microorganism, an animal cell or a plant cell, which comprises a recombinant vector into which a DNA encoding an

immunologically functional molecule is inserted, in accordance with a general culturing method, to thereby produce and accumulate the immunologically functional molecule, and then recovering the immunologically functional molecule from the culture.

As the method for expressing the gene, secretion production, expression of a fusion protein and the like can be carried out in accordance with the method described in *Molecular Cloning*, Second Edition, in addition to the direct expression.

Examples of the method for producing an immunologically functional molecule include a method of intracellular expression in a host cell, a method of extracellular secretion from a host cell, and a method of production on a host cell membrane outer envelope. The method can be selected by changing the host cell used or the structure of the immunologically functional molecule produced.

(0135)

When the immunologically functional molecule of the present invention is produced in a host cell or on a host cell membrane outer envelope, it can be positively secreted extracellularly in accordance with the method of Paulson et al. [*J. Biol. Chem.*, **264**, 17619 (1989)], the method of Lowe et al. [*Proc. Natl. Acad. Sci. USA*, **86**, 8227 (1989), *Genes Develop.*, **4**, 1288 (1990)], the methods described in Japanese Published Examined Patent Application No. 336963/93 and Japanese Published Examined Patent Application No. 823021/94 and the like.

That is, an antibody molecule of interest can be positively secreted extracellularly from a host cell by inserting a DNA encoding the antibody molecule and a DNA encoding a signal peptide suitable for the expression of the antibody molecule into an expression vector using a gene recombination technique, introducing the expression

vector into the host cell and then expressing the antibody molecule.

Also, its production amount can be increased in accordance with the method described in Japanese Published Examined Patent Application No. 227075/90 using a gene amplification system using a dihydrofolate reductase gene.

In addition, the immunologically functional molecule can also be produced using a gene-introduced animal individual (transgenic non-human animal) or a plant individual (transgenic plant) which is constructed by the redifferentiation of an animal or plant cell into which the gene is introduced.

(0136)

When the transformant is an animal individual or a plant individual, an immunologically functional molecule can be produced in accordance with a general method by rearing or cultivating it to thereby produce and accumulate the immunologically functional molecule and then recovering the immunologically functional molecule from the animal or plant individual.

Examples of the method for producing an immunologically functional molecule using an animal individual include a method in which the immunologically functional molecule of interest is produced in an animal constructed by introducing a gene in accordance with a known method [*American Journal of Clinical Nutrition*, 63, 627S (1996); *Bio/Technology*, 9, 830 (1991)].

(0137)

In the case of an animal individual, an immunologically functional molecule can be produced by rearing a transgenic non-human animal into which a DNA encoding an antibody molecule is introduced to thereby produce and accumulate the immunologically functional molecule in the animal, and then recovering the immunologically functional molecule from the animal.

Examples of the place of the animal where the composition is produced and accumulated include milk (Japanese Published Examined Patent Application No. 309192/88) and eggs of the animal. As the promoter used in this case, any promoter can be used, so long as it can function in an animal. Preferred examples include mammary gland cell-specific promoters such as  $\alpha$  casein promoter,  $\beta$  casein promoter,  $\beta$  lactoglobulin promoter, whey acidic protein promoter and the like.

(0138)

Example of the method for producing an immunologically functional molecule using a plant individual include a method in which an immunologically functional molecule is produced by cultivating a transgenic plant into which a DNA encoding an antibody molecule is introduced by a known method [*Tissue Culture*, 20 (1994); *Tissue Culture*, 21 (1995); *Trends in Biotechnology*, 15, 45 (1997)] to produce and accumulate the immunologically functional molecule in the plant, and then recovering the immunologically functional molecule from the plant.

(0139)

Regarding purification of an immunologically functional molecule produced by a transformant into which a gene encoding an antibody molecule is introduced, for example, when the immunologically functional molecule is intracellularly expressed in a dissolved state, the cells after culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonic oscillator, French press, Manton Gaulin homogenizer, dymomill or the like to obtain a cell-free extract. A purified product of the immunologically functional molecule can be obtained from a supernatant obtained by centrifuging the cell-free extract, by using a general enzyme isolation purification techniques such as solvent extraction; salting out; desalting with ammonium sulfate, etc.; precipitation

with an organic solvent; anion exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical), etc.; cation exchange chromatography using a resin such as S-Sepharose FF (manufactured by Pharmacia), etc.; hydrophobic chromatography using a resin such as butyl-Sepharose, phenyl-Sepharose, etc.; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis such as isoelectric focusing, etc.; and the like which may be used alone or in combination.

(0140)

Also, when the immunologically functional molecule is expressed intracellularly by forming an insoluble body, the cells are recovered, disrupted and centrifuged in the same manner, and the insoluble body of the immunologically functional molecule is recovered as a precipitation fraction. The recovered insoluble body of the immunologically functional molecule is solubilized using a protein denaturing agent. The immunologically functional molecule is made into a normal three-dimensional structure by diluting or dialyzing the solubilized solution, and then a purified product of the immunologically functional molecule is obtained by the same isolation purification method.

When the immunologically functional molecule is secreted extracellularly, the immunologically functional molecule or derivatives thereof can be recovered from the culture supernatant. That is, the culture is treated by a technique such as centrifugation or the like to obtain a soluble fraction, and a purified preparation of the immunologically functional molecule can be obtained from the soluble fraction by the same isolation purification method.

Examples of the thus obtained immunologically functional molecule include an antibody, the fragment of

the antibody, a fusion protein comprising the Fc region of the antibody, and the like.

(0141)

As an example for obtaining the immunologically functional molecule, a method for producing a composition of a humanized antibody is described below in detail, but other immunologically functional molecules can also be obtained in a manner similar to the method.

(1) Construction of vector for humanized antibody expression

A vector for humanized antibody expression is an expression vector for animal cell into which genes encoding the heavy chain (H chain) and light chain (L chain) C regions of a human antibody are inserted, which can be constructed by cloning each of genes encoding the H chain and L chain C regions of a human antibody into an expression vector for animal cell.

The C regions of a human antibody may be the H chain and L chain of any human antibody. Examples include the C region belonging to IgG1 subclass in the H chain of a human antibody (hereinafter referred to as "hC<sub>H1</sub>"), the C region belonging to  $\kappa$  class in the L chain of a human antibody (hereinafter referred to as "hC <sub>$\kappa$</sub> "), and the like.

(0142)

As the genes encoding the H chain and L chain C regions of a human antibody, a chromosomal DNA comprising an exon and an intron can be used or a cDNA can also be used.

As the expression vector for animal cell, any vector can be used, so long as a gene encoding the C region of a human antibody can be inserted thereinto and expressed therein. Examples include pAGE107 [Cytotechnology, 3, 133 (1990)], pAGE103 [J. Biochem., 101, 1307 (1987)], pHSG274 [Gene, 27, 223 (1984)], pKCR [Proc. Natl. Acad. Sci. USA, 78, 1527 (1981)], pSG1  $\beta$  d2-4 [Cytotechnology, 4, 173

(1990)] and the like. Examples of the promoter and enhancer in the expression vector for animal cell include SV40 early promoter and enhancer [*J. Biochem.*, 101, 1307 (1987)], Moloney mouse leukemia virus LTR promoter [*Biochem. Biophys. Res. Commun.*, 149, 960 (1987)], immunoglobulin H chain promoter [*Cell*, 41, 479 (1985)] and enhancer [*Cell*, 33, 717 (1983)], and the like.

(0143)

The humanized antibody expression vector may be either of a type in which genes encoding the H chain and L chain of an antibody exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression amounts of the H and L chains of an antibody in animal cells, a tandem type of the humanized antibody expression vector is more preferred [*J. Immunol. Methods*, 167, 271 (1994)].

The constructed humanized antibody expression vector can be used for expression of a human chimeric antibody and a human CDR-grafted antibody in animal cells.

(0144)

(2) Preparation of cDNA encoding V region of antibody derived from animal other than human

cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human, such as a mouse antibody, can be obtained in the following manner.

A cDNA is synthesized by extracting mRNA from a hybridoma cell which produces the mouse antibody of interest. The synthesized cDNA is cloned into a vector such as a phage or a plasmid to obtain a cDNA library. Each of a recombinant phage or recombinant plasmid comprising a cDNA encoding the H chain V region and a recombinant phage or recombinant plasmid comprising a cDNA

encoding the L chain V region is isolated from the library using a C region part or a V region part of an existing mouse antibody as the probe. Full nucleotide sequences of the H chain and L chain V regions of the mouse antibody of interest on the recombinant phage or recombinant plasmid are determined, and full amino acid sequences of the H chain and L chain V regions are deduced from the nucleotide sequences.

As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used so long as a hybridoma cell can be produced therefrom.

(0145)

Examples of the method for preparing total RNA from a hybridoma cell include the guanidine thiocyanate-cesium trifluoroacetate method [*Methods in Enzymology*, 154, 3 (1987)] and the like, and examples of the method for preparing mRNA from total RNA, an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, Second Edition) and the like. In addition, examples of a kit for preparing mRNA from a hybridoma cell include Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) and the like.

(0146)

Examples of the method for synthesizing cDNA and preparing a cDNA library include the usual methods (*Molecular Cloning*, Second Edition, *Current Protocols in Molecular Biology*, Supplement 1-34), methods using a commercially available kit such as SuperScript™, Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by GIBCO BRL) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene), and the like.

(0147)

In preparing the cDNA library, the vector into which a cDNA synthesized using mRNA extracted from a hybridoma cell as the template is inserted may be any

vector so long as the cDNA can be inserted. Examples include ZAP Express [*Strategies*, 5, 58 (1992)], pBluescript II SK(+) [*Nucleic Acids Research*, 17, 9494 (1989)],  $\lambda$ zapII (manufactured by Stratagene),  $\lambda$ gt10 and  $\lambda$ gt11 [*DNA Cloning, A Practical Approach*, I, 49 (1985)], Lambda BlueMid (manufactured by Clontech),  $\lambda$ ExCell, pT7T3 18U (manufactured by Pharmacia), pcD2 [*Mol. Cell. Biol.*, 3, 280 (1983)], pUC18 [*Gene*, 33, 103 (1985)] and the like.

(0148)

As *Escherichia coli* into which the cDNA library constructed from a phage or plasmid vector is introduced, any *Escherichia coli* can be used, so long as the cDNA library can be introduced, expressed and maintained. Examples include XL1-Blue MRF' [*Strategies*, 5, 81 (1992)], C600 [*Genetics*, 32, 440 (1954)], Y1088 and Y1090 [*Science*, 222, 778 (1983)], NM522 [*J. Mol. Biol.*, 166, 1 (1983)], K802 [*J. Mol. Biol.*, 16, 118 (1966)], JM105 [*Gene*, 38, 275 (1985)] and the like.

(0149)

As the method for selecting a cDNA clone encoding the H chain and L chain V regions of an antibody derived from an animal other than human from the cDNA library, a colony hybridization or a plaque hybridization using an isotope- or fluorescence-labeled probe can be used (*Molecular Cloning*, Second Edition). The cDNA encoding the H chain and L chain V regions can also be prepared by preparing primers and carrying out polymerase chain reaction (hereinafter referred to as "PCR"; *Molecular Cloning*, Second Edition; *Current Protocols in Molecular Biology*, Supplement 1-34) using a cDNA synthesized from mRNA or a cDNA library as the template.

(0150)

The nucleotide sequences of the cDNAs can be determined by digesting the selected cDNAs with appropriate restriction enzymes, cloning the fragments into a plasmid

such as pBluescript SK(-) (manufactured by Stratagene) or the like, carrying out the reaction of a generally used nucleotide sequence analyzing method such as the dideoxy method [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] of Sanger et al. or the like and then analyzing the clones using an automatic nucleotide sequence analyzer such as A.L.F. DNA Sequencer (manufactured by Pharmacia) or the like.

(0151)

Whether or not the obtained cDNAs are encoding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a secretory signal sequence can be confirmed by deducing the full amino acid sequences of the H chain and L chain V regions from the determined nucleotide sequence and comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)].

(0152)

(3) Analysis of amino acid sequence of V region of antibody derived from animal other than human

Regarding the full amino acid sequences of the H chain and L chain V regions of the antibody containing a secretory signal sequence, the length of the secretory signal sequence and the N-terminal amino acid sequences can be deduced and subgroups to which they belong can also be found, by comparing them with the full amino acid sequences of the H chain and L chain V regions of known antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)]. In addition, the amino acid sequences of the H chain and L chain V regions of each CDR can also be found by comparing them with the amino acid sequences of the H chain and L chain V regions of known

antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services, (1991)].

(0153)

(4) Construction of human chimeric antibody expression vector

A human chimeric antibody expression vector can be constructed by cloning cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human into upstream of genes encoding the H chain and L chain C regions of a human antibody in the vector for humanized antibody expression constructed in the item 3(1). For example, a human chimeric antibody expression vector can be constructed by linking each of cDNAs encoding the H chain and L chain V regions of an antibody derived from an animal other than human to a synthetic DNA comprising nucleotide sequences at the 3'-terminals of the H chain and L chain V regions of an antibody derived from an animal other than human and nucleotide sequences at the 5'-terminals of the H chain and L chain C regions of a human antibody and also having a recognition sequence of an appropriate restriction enzyme at both terminals, and by cloning them into upstream of genes encoding the H chain and L chain C regions of a human antibody contained in the vector for humanized antibody expression constructed described in the item 3(1).

(0154)

(5) Construction of cDNA encoding V region of human CDR-grafted antibody

cDNAs encoding the H chain and L chain V regions of a human CDR-grafted antibody can be obtained as follows. First, amino acid sequences of the frameworks (hereinafter referred to as "FR") of the H chain and L chain V regions of a human antibody for grafting CDR of the H chain and L chain V regions of an antibody derived from an animal other than human is selected. As the amino acid sequences of FRs

of the H chain and L chain V regions of a human antibody, any amino acid sequences can be used so long as they are derived from a human antibody. Examples include amino acid sequences of FRs of the H chain and L chain V regions of human antibodies registered at databases such as Protein Data Bank, etc., amino acid sequences common in each subgroup of FRs of the H chain and L chain V regions of human antibodies [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)] and the like. But in order to produce a human CDR-grafted antibody having potent activity, it is preferable to select an amino acid sequence having a homology as high as possible (at least 60% or more) with amino acid sequences of the H chain and L chain V regions of an antibody of interest derived from an animal other than human.

(0155)

Next, the amino acid sequences of CDRs of the H chain and L chain V regions of the antibody of interest derived from an animal other than human are grafted to the selected amino acid sequences of FRs of the H chain and L chain V regions of a human antibody to design amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody. The designed amino acid sequences are converted into DNA sequences by considering the frequency of codon usage found in nucleotide sequences of antibody genes [*Sequences of Proteins of Immunological Interest*, US Dep. Health and Human Services (1991)], and the DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the human CDR-grafted antibody are designed. Based on the designed DNA sequences, several synthetic DNA fragments having a length of about 100 bases are synthesized, and PCR is carried out using them. In this case, it is preferable in each of the H chain and the L chain that 6 synthetic DNAs are designed in

view of the reaction efficiency of PCR and the lengths of DNAs which can be synthesized.

(0156)

Also, they can be easily cloned into the vector for humanized antibody expression constructed in the item 3(1) by introducing recognition sequences of an appropriate restriction enzyme into the 5'-terminals of the synthetic DNA present on both terminals. After the PCR, the amplified product is cloned into a plasmid such as pBluescript SK(-) (manufactured by Stratagene) or the like and the nucleotide sequences are determined by the method in the item 3(2) to thereby obtain a plasmid having DNA sequences encoding the amino acid sequences of the H chain and L chain V regions of the desired human CDR-grafted antibody.

(0157)

(6) Construction of human CDR-grafted antibody expression vector

A human CDR-grafted antibody expression vector can be constructed by cloning the cDNAs encoding the H chain and L chain V regions of the human CDR-grafted antibody constructed in the item 3(5) into upstream of the gene encoding H chain and L chain C regions of a human antibody in the vector for humanized antibody expression described in the item 3(1). For example, the human CDR-grafted antibody expression vector can be constructed by introducing recognizing sequences of an appropriate restriction enzyme into the 5'-terminals of both terminals of a synthetic DNA fragment, among the synthetic DNA fragments which are used when PCR is carried out in the item 3(5) for constructing the H chain and L chain V regions of the human CDR-grafted antibody, so that they are cloned into upstream of the genes encoding the H chain and L chain C regions of a human antibody in the vector for

humanized antibody expression described in the item 3(1) in such a manner that they can be expressed in a suitable form. (0158)

(7) Stable production of humanized antibody

A transformant capable of stably producing a human chimeric antibody and a human CDR-grafted antibody (both hereinafter referred to as "humanized antibody") can be obtained by introducing the humanized antibody expression vectors described in the items 3(4) and (6) into an appropriate animal cell.

Examples of the method for introducing a humanized antibody expression vector into an animal cell include electroporation [Japanese Published Examined Patent Application No. 257891/90, *Cytotechnology*, 3, 133 (1990)] and the like.

As the animal cell into which a humanized antibody expression vector is introduced, any cell can be used so long as it is an animal cell which can produce the humanized antibody.

Examples include mouse myeloma cells such as NSO cell and SP2/0 cell, Chinese hamster ovary cells such as CHO/dhfr<sup>-</sup> cell and CHO/DG44 cell, rat myeloma such as YB2/0 cell and IR983F cell, BHK cell derived from a syrian hamster kidney, a human myeloma cell such as Namalwa cell, and the like, and a Chinese hamster ovary cell CHO/DG44 cell, a rat myeloma YB2/0 cell and the host cells of the present invention described in the item 5 are preferred.

(0159)

After introduction of the humanized antibody expression vector, a transformant capable of stably producing the humanized antibody can be selected using a medium for animal cell culture comprising an agent such as G418 sulfate (hereinafter referred to as "G418"; manufactured by SIGMA) and the like in accordance with the method disclosed in Japanese Published Examined Patent

Application No. 257891/90. Examples of the medium for animal cell culture include RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nihon Pharmaceutical), EX-CELL 302 medium (manufactured by JRH), IMDM medium (manufactured by GIBCO BRL), Hybridoma-SFM medium (manufactured by GIBCO BRL) media obtained by adding various additives such as fetal bovine serum (hereinafter referred to as "FBS") to these media, and the like. The humanized antibody can be produced and accumulated in the culture supernatant by culturing the obtained transformant in a medium. The expression level and antigen binding activity of the humanized antibody in the culture supernatant can be measured by a method such as enzyme-linked immunosorbent assay [hereinafter referred to as "ELISA"; *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like. Also, the expression level of the humanized antibody by the transformant can be increased using a DHFR gene amplification system in accordance with the method disclosed in Japanese Published Examined Patent Application No. 257891/90.

(0160)

The humanized antibody can be purified from a culture supernatant of the transformant using a protein A column [*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 8 (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)]. In addition, purification methods generally used for the purification of proteins can also be used. For example, the purification can be carried out through the combination of a gel filtration, an ion exchange chromatography and an ultrafiltration. The molecular weight of the H chain, L chain or antibody molecule as a whole of the purified humanized antibody can be measured, e.g., by polyacrylamide

gel electrophoresis [hereinafter referred to as "SDS-PAGE"; *Nature*, 227, 680 (1970)], Western blotting [*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 12, (1988), *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)] or the like.

Thus, methods for producing an immunologically functional molecule using an animal cell as the host have been described, but, as described above, the immunologically functional molecule can also be produced by a yeast, an insect cell, a plant cell, an animal individual or a plant individual by the same methods on the animal cell.

(0161)

#### 4. Production of immunologically functional molecule using cell of the present invention

The immunologically functional molecule of the present invention can be produced by preparing a cell expressing an antibody molecule using the method described in the item 1, culturing the cell using the method for preparing the immunologically functional molecule described in the item 3 and then purifying the immunologically functional molecule of interest from the resulting culture.

When a host cell has the ability to express an antibody molecule innately, it is not introduce the immunologically functional molecule of the present invention into the cell, and the immunologically functional molecule can be produced by preparing a cell expressing an antibody molecule using host cell as the parent cell line according to the method described in the item 1, culturing the cell using the method for preparing the immunologically functional molecule described in the item 3 and then purifying the immunologically functional molecule of interest from the resulting culture.

(0162)

#### 45. Activity evaluation of immunologically functional molecule

As the method for measuring the amount of the purified immunologically functional molecule, the activity to bind to an antibody and the effector function of the purified immunologically functional molecule, the known method described in *Monoclonal Antibodies, Antibody Engineering* and the like can be used. As the examples, when the immunologically functional molecule is a humanized antibody, the binding activity with an antigen and the binding activity with an antigen-positive cultured cell line can be measured by methods such as ELISA, an immunofluorescent method [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like. The cytotoxic activity against an antigen-positive cultured cell line can be evaluated by measuring CDC activity, ADCC activity [*Cancer Immunol. Immunother.*, 36, 373 (1993)] and the like. Also, safety and therapeutic effect of the immunologically functional molecule in human can be evaluated using an appropriate model of animal species relatively close to human, such as *Macaca fascicularis* or the like.

(0163)

#### 6. Analysis of sugar chains binding to antibody molecules expressed in various cells

The sugar chain structure binding to an antibody molecule expressed in various cells can be analyzed in accordance with the general analysis of the sugar chain structure of a glycoprotein. For example, the sugar chain which is bound to IgG molecule comprises a neutral sugar such as galactose, mannose, fucose or the like, an amino sugar such as *N*-acetylglucosamine or the like and an acidic sugar such as sialic acid or the like, and can be analyzed by a method such as a sugar chain structure analysis or the like using sugar composition analysis, two dimensional sugar chain mapping or the like.

(0164)

(1) Analysis of neutral sugar and amino sugar compositions

The sugar chain composition binding to an antibody molecule can be analyzed by carrying out acid hydrolysis of sugar chains with an acid such as trifluoroacetic acid or the like to release a neutral sugar or an amino sugar and measuring the composition ratio.

Example include a method using a sugar composition analyzer (BioLC) manufactured by Dionex. The BioLC is an apparatus which analyzes a sugar composition by HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection) [*J. Liq. Chromatogr.*, **6**, 1577 (1983)].

The composition ratio can also be analyzed by a fluorescence labeling method using 2-aminopyridine. Specifically, the compositional ratio can be calculated in accordance with a known method [*Agric. Biol. Chem.*, **55**(1); 283-284 (1991)], by labeling an acid-hydrolyzed sample with a fluorescence with 2-aminopyridylation and then analyzing the composition by HPLC.

(0165)

(2) Analysis of sugar chain structure

The sugar chain structure binding to an antibody molecule can be analyzed by the two dimensional sugar chain mapping method [*Anal. Biochem.*, **171**, 73 (1988), *Biochemical Experimentation Methods 23 - Methods for Studying Glycoprotein Sugar Chains* (Japan Scientific Societies Press) edited by Reiko Takahashi (1989)]. The two dimensional sugar chain mapping method is a method for deducing a sugar chain structure by, e.g., plotting the retention time or elution position of a sugar chain by reverse phase chromatography as the X axis and the retention time or elution position of the sugar chain by normal phase chromatography as the Y axis, respectively, and comparing them with such results of known sugar chains.

(0166)

Specifically, sugar chains are released from an antibody by subjecting the antibody to hydrazinolysis, and the released sugar chain is subjected to fluorescence labeling with 2-aminopyridine (hereinafter referred to as "PA") [*J. Biochem.*, 95, 197 (1984)], and then the sugar chains are separated from an excess PA-treating reagent by gel filtration, and subjected to reverse phase chromatography. Thereafter, each peak of the separated sugar chains are subjected to normal phase chromatography. The sugar chain structure can be deduced by plotting the results on a two dimensional sugar chain map and comparing them with the spots of a sugar chain standard (manufactured by Takara Shuzo) or a literature [*Anal. Biochem.*, 171, 73 (1988)].

The structure deduced by the two dimensional sugar chain mapping method can be confirmed by further carrying out mass spectrometry such as MALDI-TOF-MS of each sugar chain or the like.

(0167)

7. Comparison of activity of enzyme relating to modification of sugar chain in various host cells

The activity of an enzyme relating to the modification of a sugar chain, that is, a glycosyltransferase and a glycolytic enzyme, in various host cells can be carried out by biochemical methods or genetic engineering techniques described in *New Biochemical Experimentation Series 3-Saccharides I, Glycoprotein* (Tokyo Kagaku Dojin), edited by Japanese Biochemical society (1988); *Cell Engineering, Supplement, Experimental Protocol Series, Glycobiology Experimental Protocol, Glycoprotein, Glycolipid and Proteoglycan* (Shujun-sha), edited by Naoyuki Taniguchi, Akemi Suzuki, Kiyoshi Furukawa and Kazuyuki Sugawara (1996);, *Molecular Cloning, Second Edition; Current Protocols in Molecular Biology*; and the like.

Examples of the biochemical method include a method in which the enzyme activity is evaluated using an enzyme-specific substrate and the like. Examples of the genetic engineering technique include the Northern analysis, RT-PCR and the like which measures the amount of mRNA of a gene encoding the enzyme.

(0186)

#### 8. Immunological determination method for discriminating sugar chain structure of antibody molecule

An immunologically functional molecule comprises an antibody molecule in which sugar chains binding to the Fc region of the antibody are different in structure. The immunologically functional molecule in which the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain is 20% or more among the total complex N-glycoside-linked sugar chains binding to the Fc region in the immunologically functional molecule reducing end has potent ADCC activity. The immunologically functional molecule can be identified by using the method for analyzing the sugar chain structure of an antibody molecule described in the item 6. Also, it can also be identified by an Immunological determination method using a lectin.

The sugar chain structure of an antibody molecule can be identified by the Immunological determination method using a lectin in accordance with the known Immunological determination method such as Western staining, IRA (radioimmunoassay), VIA (viroimmunoassay), EIA (enzymimmunoassay), FIA (fluoroimmunoassay), MIA (metalloimmunoassay) and the like described in *Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, Inc. (1995); *Immunoassay*, 3rd Ed., Igakushoin (1987); *Enzyme Antibody Method*, Revised Edition, Gakusai Kikaku (1985); and the like.

A lectin which recognizes the sugar chain structure of an antibody molecule comprised in an immunologically functional molecule is labeled, and the labeled lectin is allowed to react with an immunologically functional molecule which is a sample. Then, the amount of the complex of the labeled lectin with the antibody molecule is measured.

Examples of the lectin used for identifying the sugar chain structure of an antibody molecule include WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (concanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (*Aleuria aurantia* lectin), ACL (*Amaranthus caudatus* lectin), BPL (*Bauhinia purpurea* lectin), DSL (*Datura stramonium* lectin), DBA (*Dolichos biflorus* agglutinin), EBL (elderberry bark lectin), ECL (*Erythrina cristagalli* lectin), EEL (*Euonymus europaeus* lectin), GNL (*Galanthus nivalis* lectin), GSL (*Griffonia simplicifolia* lectin), HPA (*Helix pomatia* agglutinin), HHL (*Hippeastrum hybrid* lectin), Jacalin, LTL (*Lotus tetragonolobus* lectin), LEL (*Lycopersicon esculentum* lectin), MAL (*Maackia amurensis* lectin), MPL (*Maclura pomifera* lectin), NPL (*Narcissus pseudonarcissus* lectin), PNA (peanut agglutinin), E-PHA (*Phaseolus vulgaris* erythroagglutinin), PTL (*Psophocarpus tetragonolobus* lectin), RCA (*Ricinus communis* agglutinin), STL (*Solanum tuberosum* lectin), SJA (*Sophora japonica* agglutinin), SBA (soybean agglutinin), UEA (*Ulex europaeus* agglutinin), VVL (*Vicia villosa* lectin) and WFA (*Wisteria floribunda* agglutinin).

It is preferable to use a lectin which specifically recognizes a sugar chain structure wherein fucose binds to the *N*-acetylglucosamine in the reducing end in the complex *N*-glycoside-linked sugar chain. Examples include *Lens*

*culinaris* lectin LCA (lentil agglutinin derived from *Lens culinaris*), pea lectin PSA (pea lectin derived from *Pisum sativum*), broad bean lectin VFA (agglutinin derived from *Vicia faba*) and *Aleuria aurantia* lectin AAL (lectin derived from *Aleuria aurantia*).

(0186)

8. Method for preparing medicament having desired effector function by changing ratio of *N*-glycoside-linked sugar chain in which sugar is not bound to *N*-acetylglucamine in reducing end in composition comprising immunologically functional molecule having *N*-glycoside-linked sugar chain

The immunologically functional molecule having an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end can be prepared, for example, by the production method of the immunologically functional molecule of the present invention described in the item 4, and a medicament having a desired effector function can be prepared by changing the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end using the obtained a composition comprising the immunologically functional molecule as a material. By changing the ratio of the *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end, for example, an immunologically functional molecular composition having an *N*-glycoside-linked sugar chain in which a sugar is not bound to *N*-acetylglucosamine in the sugar chain reducing end can be purified, and a medicament composition having any ratio by adding the purified immunologically functional molecule composition.

(0169)

9. Method for screening cell in which activity of enzyme relating to sugar chain in glycoprotein is controlled by artificial technique

The screening method of the present invention comprises selecting a desired mutant as a measure of the activity of an enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein.

Specifically, the mutant can be screened as a measure of the activity of an enzyme relating to the modification of a glycoprotein or the sugar chain structure of a glycoprotein, and, for example, the desired mutant is selected by the method described in the item 1(2).

(0170)

#### 10. Application of immunologically functional molecule of the present invention

The immunologically functional molecule of the present invention has potent antibody-dependent cell-mediated cytotoxic activity. An antibody having potent antibody-dependent cell-mediated cytotoxic activity is useful for preventing and treating various diseases including cancers, inflammatory diseases, immune diseases such as autoimmune diseases, allergies and the like, circulatory organ diseases and viral or bacterial infections.

In the case of cancers, namely malignant tumors, cancer cells grow. General anti-tumor agents inhibit the growth of cancer cells. In contrast, an antibody having potent antibody-dependent cell-mediated cytotoxic activity can treat cancers by injuring cancer cells through its cell killing effect, and therefore, it is more effective as a therapeutic agent than the general anti-tumor agents. At present, in the therapeutic agent for cancers, an anti-tumor effect of an antibody medicament alone is insufficient so that combination therapy with chemotherapy has been carried out [*Science*, 280, 1197 (1998)]. If more potent anti-tumor effect is found by the immunologically functional molecule of the present invention alone, the

dependency on chemotherapy will be decreased and side effects will be reduced.

(0171)

In allergies, *in vivo* reactions of the diseases are induced by the release of a mediator molecule by immunocytes, so that the allergy reaction can be inhibited by eliminating immunocytes using an antibody having potent ADCC or CDC activity.

(0172)

Examples of the circulatory organ diseases include arteriosclerosis and the like. The arteriosclerosis is treated using balloon catheter at present, but circulatory organ diseases can be prevented and treated by inhibiting growth of arterial cells in resticture after treatment using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

(0173)

Various diseases including viral and bacterial infections can be prevented and treated by inhibiting proliferation of cells infected with a virus or bacterium using an antibody having potent ADCC or CDC activity.

Also, an antibody in which ADCC or CDC activity is inhibited is useful for preventing or treating autoimmune diseases in view of that the accelerated immune reaction in the autoimmune diseases can be inhibited. Also, they are useful for immunosuppressant agents, inhibition of unnecessary cell death, prevention and treatment of blood cancer. Furthermore, in pharmaceuticals in which activity other than the effector function of the immunologically functional molecule, for example, neutralizing activity against a recognized antigen molecule, side effects by potent effector function can be inhibited.

(0174)

The medicament comprising the immunologically functional molecule (antibody, etc.) of the present

invention can be administered as a therapeutic agent alone, but generally, it is preferable to provide it as a pharmaceutical formulation produced by an appropriate method well known in the technical field of manufacturing pharmacy, by mixing it with at least one pharmaceutically acceptable carrier.

It is desirable to select a route of administration which is most effective in treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular, intravenous or the like. In an antibody preparation, intravenous administration is preferable.

The dosage form includes sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

Examples of the pharmaceutical preparation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

(0175)

Liquid preparations, such as emulsions and syrups, can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose, etc.; glycols, such as polyethylene glycol, propylene glycol, etc.; oils, such as sesame oil, olive oil, soybean oil, etc.; antiseptics, such as p-hydroxybenzoic acid esters, etc.; flavors, such as strawberry flavor, peppermint, etc.; and the like.

Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, etc.; disintegrating agents, such as starch, sodium alginate, etc.; lubricants, such as magnesium stearate, talc, etc.; binders, such as polyvinyl alcohol, hydroxypropylcellulose, gelatin, etc.; surfactants, such as fatty acid ester, etc.; plasticizers, such as glycerine, etc.; and the like.

Examples of the pharmaceutical preparation suitable for parenteral administration include injections, suppositories, sprays and the like.

Injections may be prepared using a carrier, such as a salt solution, a glucose solution, a mixture of both thereof or the like. Also, powdered injections can be prepared by freeze-drying the immunologically functional molecule in the usual way and adding sodium chloride thereto.

(0176)

Suppositories may be prepared using a carrier such as cacao butter, hydrogenated fat, carboxylic acid or the like.

Also, sprays may be prepared using the immunologically functional molecule as such or using a carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the immunologically functional molecule by dispersing it as fine particles.

Examples of the carrier include lactose, glycerol and the like. Depending on the properties of the immunologically functional molecule and the carrier, it is possible to produce pharmaceutical preparations such as aerosols, dry powders and the like. In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

(0177)

Although the clinical dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10  $\mu\text{g/kg}$  to 20  $\text{mg/kg}$  per day and per adult.

Also, as the method for examining antitumor effect of the immunologically functional molecule against various tumor cells, *in vitro* tests include CDC activity measuring

method, ADCC activity measuring method and the like, and *in vivo* tests include antitumor experiments using a tumor system in an experimental animal such as a mouse, etc. and the like.

CDC activity and ADCC activity measurements and antitumor experiments can be carried out in accordance with the methods described in *Cancer Immunology Immunotherapy*, 36, 373 (1993); *Cancer Research*, 54, 1511 (1994) and the like.

The present invention will be described below in detail based on Examples; however, Examples are only simple illustrations, and the scope of the present invention is not limited thereto.

(0178)

(Examples)

Example 1

Production of anti-ganglioside GD3 human chimeric antibody:  
1. Construction of tandem expression vector pChiLHGM4 for anti-ganglioside GD3 human chimeric antibody

A plasmid pChi641LGM40 was constructed by ligating a fragment of about 4.03 kb containing an L chain cDNA, obtained by digesting an L chain expression vector, pChi641LGM4 [*J. Immunol. Methods*, 167, 271 (1994)] for anti-ganglioside GD3 human chimeric antibody (hereinafter referred to as "anti-GD3 chimeric antibody") with restriction enzymes *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (manufactured by Takara Shuzo) with a fragment of about 3.40 kb containing a G418-resistant gene and a splicing signal, obtained by digesting an expression vector pAGE107 [*Cytotechnology*, 3, 133 (1990)] for animal cell with restriction enzymes *Mlu*I (manufactured by Takara Shuzo) and *Sal*I (manufactured by Takara Shuzo) using DNA Ligation Kit (manufactured by Takara Shuzo), and then transforming *E. coli* HB101 (*Molecular Cloning*, Second Edition) with the ligated product.

(0179)

Next, a fragment of about 5.68 kb containing an L chain cDNA, obtained by digesting the constructed plasmid pChi641LGM40 with a restriction enzyme *Cla*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (manufactured by Takara Shuzo), was ligated with a fragment of about 8.40 kb containing an H chain cDNA, obtained by digesting an anti-GD3 chimeric antibody H chain expression vector, pChi641HGM4 [*J. Immunol. Methods*, 167, 271 (1994)] with a restriction enzyme, *Xho*I (manufactured by Takara Shuzo), blunt-terminating it using DNA Blunting Kit (manufactured by Takara Shuzo) and further digesting it with *Mlu*I (manufactured by Takara Shuzo), using DNA Ligation Kit (manufactured by Takara Shuzo), and then *E. coli* HB101 (*Molecular Cloning*, Second Edition) was transformed with the ligated product to thereby construct a tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody.

(0180)

## 2. Preparation of cells stably producing anti-GD3 chimeric antibody

Cells capable of stably producing an anti-GD3 chimeric antibody were prepared using the tandem expression vector pChi641LHGM4 for anti-GD3 chimeric antibody constructed in the item 1 of Example 1, as described below.

### (1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

After introducing 5  $\mu$ g of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into  $4 \times 10^6$  cells of rat myeloma YB2/0 [ATCC CRL-1662, J.V. Kilmarin et al., *J. Cell. Biol.*, 93, 576-582 (1982)] by electroporation [*Cytotechnology*, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) (RPMI1640 medium comprising 10% FBS (manufactured by GIBCO BRL)) and dispensed in 200

μl/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO<sub>2</sub> incubator, G418 was added to a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

(0181)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nM DHFR inhibitor, methotrexate (hereinafter referred to as "MTX"; manufactured by SIGMA) to give a density of 1 to  $2 \times 10^5$  cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO<sub>2</sub> incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants,

suitable cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0182)

The obtained anti-GD3 chimeric antibody-producing transformed cell clone 7-9-51 has been deposited on April 5, 1999, as FERM BP-6691 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(0183)

(2) Preparation of antibody-producing cell using CHO/DG44 cell

After introducing 4  $\mu$ g of the anti-GD3 chimeric antibody expression vector, pChi641LHGM4, into  $1.6 \times 10^6$  cells of CHO/DG44 [G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA*, 77, 4216-4220 (1980)] by electroporation [Cytotechnology, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) [IMDM medium comprising 10% FBS and 1x concentration of HT supplement (manufactured by GIBCO BRL)] and dispensed in 200  $\mu$ l/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them at 37°C for 24 hours in a 5% CO<sub>2</sub> incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

(0184)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an IMDM-dFBS(10) medium [IMDM medium comprising 10% dialyzed fetal bovine serum (hereinafter referred to as "dFBS"; manufactured by GIBCO BRL)] comprising 0.5 mg/ml G418 and 10 nM MTX to give a density of 1 to  $2 \times 10^5$  cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO<sub>2</sub> incubator. Regarding the transformants in wells in which their growth was observed, the MTX concentration was increased to 100 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 100 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount were finally obtained by the same method as described above. Among the obtained transformants, suitable cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0185)

(3) Preparation of antibody-producing cell using mouse myeloma NS0 cell

After introducing 5  $\mu$ g of the anti-GD3 chimeric antibody expression vector pChi641LHGM4 into  $4 \times 10^6$  cells of mouse myeloma NS0 by electroporation [Cytotechnology, 3, 133 (1990)], the cells were suspended in 40 ml of EX-CELL302-FBS(10) (EX-CELL302 medium comprising 10% FBS and 2

mM L-glutamine [hereinafter referred to as "L-Gln"; manufactured by GIBCO BRL)] and dispensed in 200  $\mu$ l/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO<sub>2</sub> incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-GD3 chimeric antibody in the supernatant was measured by the ELISA shown in the item 3 of Example 1.

(0186)

Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, in order to increase the amount of the antibody production using a DHFR gene amplification system, each of them was suspended in an EX-CELL302-dFBS(10) medium (EX-CELL302 medium comprising 10% dFBS and 2 mM L-Gln) comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to  $2 \times 10^5$  cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO<sub>2</sub> incubator. The antigen binding activity of the anti-GD3 chimeric antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 3 of Example 1. Regarding the transformants in wells in which production of the anti-GD3 chimeric antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the EX-CELL302-dFBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-GD3 chimeric antibody in a large amount was finally obtained by the same

method as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0187)

### 3. Measurement of binding activity of antibody to GD3 (ELISA)

The binding activity of the antibody to GD3 was measured as described below.

In 2 ml of ethanol solution containing 10  $\mu$ g of dipalmitoylphosphatidylcholine (manufactured by SIGMA) and 5  $\mu$ g of cholesterol (manufactured by SIGMA), 4 nmol of GD3 was dissolved. Into each well of a 96 well plate for ELISA (manufactured by Greiner), 20  $\mu$ l of the solution (40 pmol/well in final concentration) was dispensed, followed by air-drying, 1% bovine serum albumin (hereinafter referred to as "BSA"; manufactured by SIGMA)-containing PBS (hereinafter referred to as "1% BSA-PBS") was dispensed in 100  $\mu$ l/well, and then the reaction was carried out at room temperature for 1 hour for blocking remaining active groups. After discarding 1% BSA-PBS, a culture supernatant of a transformant or a diluted solution of a human chimeric antibody was dispensed in 50  $\mu$ l/well to carry out the reaction at room temperature for 1 hour. After the reaction, each well was washed with 0.05% Tween 20 (manufactured by Wako Pure Chemical Industries)-containing PBS (hereinafter referred to as "Tween-PBS"), a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualalex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50  $\mu$ l/well as a secondary antibody solution, and then the reaction was carried out at

room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, ABTS substrate solution [solution prepared by dissolving 0.55 g of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt in 1 liter of 0.1 M citrate buffer (pH 4.2) and adding 1 µl/ml of hydrogen peroxide to the solution just before use (hereinafter the same solution was used)] was dispensed in 50 µl/well for color development, and then absorbance at 415 nm (hereinafter referred to as "OD415") was measured. (0188)

#### 4. Purification of anti-GD3 chimeric antibody

##### (1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(1) of Example 1 was suspended in the Hybridoma-SFM medium comprising 0.2% BSA, 200 nM MTX and 100 nM triiodothyronine (hereinafter referred to as "T3"; manufactured by SIGMA) to give a density of  $3 \times 10^5$  cells/ml and cultured using a 2.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 50 rpm. After culturing them at 37°C for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named YB2/0-GD3 chimeric antibody.

(0189)

##### (2) Culturing of antibody-producing cell derived from CHO/DG44 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(2) of Example 1 was suspended in the EX-CELL302 medium comprising 3 mM L-Gln, 0.5% fatty acid concentrated solution (hereinafter

referred to as "CDLC"; manufactured by GIBCO BRL) and 0.3% Pluronic F68 (hereinafter referred to as "PF68"; manufactured by GIBCO BRL) to give a density of  $1 \times 10^6$  cells/ml, and the suspension was dispensed in 50 ml into 175 mm<sup>2</sup> flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO<sub>2</sub> incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named CHO/DG44-GD3 chimeric antibody. (0190)

(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone obtained in the item 2(3) of Example 1 was suspended in the EX-CELL302 medium comprising 2 mM L-Gln, 0.5 mg/ml G418, 200 nM MTX and 1% FBS, to give a density of  $1 \times 10^6$  cells/ml, and the suspension was dispensed in 200 ml into 175 mm<sup>2</sup> flasks (manufactured by Greiner). After culturing them at 37°C for 4 days in a 5% CO<sub>2</sub> incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (302).

Also, the transformed cell clone was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of  $3 \times 10^5$  cells/ml, and the suspension was dispensed in 200 ml into 175 mm<sup>2</sup> flasks (manufactured by Greiner). After culturing them at 37°C for 10 days in a 5% CO<sub>2</sub> incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by

Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named NS0-GD3 chimeric antibody (GIT).

(0191)

(4) Culturing of antibody-producing cell derived from SP2/0 cell and purification of antibody

The anti-GD3 chimeric antibody-producing transformed cell clone (KM-871 (FERM BP-3512)) described in Japanese Published Unexamined Patent Application No. 304989/93 (EP 533199) was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of  $3 \times 10^5$  cells/ml, and the suspension was dispensed in 200 ml into 175 mm<sup>2</sup> flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO<sub>2</sub> incubator, the culture supernatant was recovered. The anti-GD3 chimeric antibody was purified from the culture supernatant using a Prosep-A (manufactured by Bioprocessing) column in accordance with the manufacture's instructions. The purified anti-GD3 chimeric antibody was named SP2/0-GD3 chimeric antibody.

(0192)

#### 5. Analysis of purified anti-GD3 chimeric antibody

In accordance with a known method [Nature, 227, 680 (1970)], 4 µg of each of the five kinds of the anti-GD3 chimeric antibodies produced by and purified from respective animal cells, obtained in the item 4 of Example 1, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in Fig. 1. As shown in Fig. 1, a single band of about 150 kilodaltons (hereinafter referred to as "Kd") in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-GD3 chimeric antibodies. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide

sequences of H chain and L chain of the antibody (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the disulfide bond (hereinafter referred to as "S-S bond") in the molecule [Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1998); Monoclonal Antibodies: Principles and Practice, Academic Press Limited (1996)], so that it was confirmed that each anti-GD3 chimeric antibody was expressed and purified as an antibody molecule having the true structure.

(0193)

#### Example 2

Activity evaluation of anti-GD3 chimeric antibody:

##### 1. Binding activity of anti-GD3 chimeric antibody to GD3 (ELISA)

The activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1 to bind to GD3 (manufactured by Snow Brand Milk Products) was measured by the ELISA shown in the item 3 of Example 1. Fig. 2 shows a result of the examination of the binding activity measured by changing the concentration of the anti-GD3 chimeric antibody to be added. As shown in Fig. 2, the five kinds of the anti-GD3 chimeric antibodies showed almost the same binding activity to GD3. The result shows that antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods. Also, it was suggested from the comparison of the NS0-GD3 chimeric antibody (302) with the NS0-GD3 chimeric antibody (GIT) that the antigen

binding activities are constant independently of the media used in the culturing.

(0194)

## 2. *In vitro* cytotoxic activity (ADCC activity) of anti-GD3 chimeric antibody

In order to evaluate *in vitro* cytotoxic activity of the five kinds of the purified anti-GD3 chimeric antibodies obtained in the item 4 of Example 1, the ADCC activity was measured in accordance with the following method.

### (1) Preparation of target cell solution

A human melanoma cultured cell line G-361 (ATCC CRL 1424) was cultured using the RPMI1640-FBS(10) medium to prepare  $1 \times 10^6$  cells, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance  $\text{Na}_2^{51}\text{CrO}_4$  at  $37^\circ\text{C}$  for 1 hour. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, re-suspended in the medium and then incubated at  $4^\circ\text{C}$  for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After centrifugation, the precipitate was adjusted to  $2 \times 10^5$  cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(0195)

### (2) Preparation of effector cell solution

From a healthy person, 50 ml of venous blood was collected, and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to isolate a mononuclear cell layer using Lymphoprep (manufactured by Nycomed Pharma AS) in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting precipitate was re-suspended to give a density of  $2 \times 10^6$  cells/ml using the medium and used as the effector cell solution.

(0196)

(3) Measurement of ADCC activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50  $\mu$ l of the target cell solution prepared in the above (1) ( $1 \times 10^4$  cells/well) was dispensed. Next, 100  $\mu$ l of the effector cell solution prepared in the above (2) was added thereto ( $2 \times 10^5$  cells/well, the ratio of effector cells to target cells becomes 20:1). Subsequently, each of the anti-GD3 chimeric antibodies was added to give a final concentration from 0.0025 to 2.5  $\mu$ g/ml, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, and the amount of  $^{51}\text{Cr}$  in the supernatant was measured using a  $\gamma$ -counter. The amount of spontaneously released  $^{51}\text{Cr}$  was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution, and measuring the amount of  $^{51}\text{Cr}$  in the supernatant. The amount of total released  $^{51}\text{Cr}$  was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N hydrochloric acid instead of the effector cell solution, and measuring the amount of  $^{51}\text{Cr}$  in the supernatant. The ADCC activity was calculated from the following equation (I):

(0197)

Equation (I)

$$\text{ADCC activity (\%)} = \frac{{}^{51}\text{Cr in sample supernatant} - \text{spontaneously released } {}^{51}\text{Cr}}{\text{total released } {}^{51}\text{Cr} - \text{spontaneously released } {}^{51}\text{Cr}} \times 100 \text{ (II)}$$

(0198)

The results are shown in Fig. 3. As shown in Fig. 3, among the five kinds of the anti-GD3 chimeric antibodies, the YB2/0-GD3 chimeric antibody showed the most potent ADCC activity, followed by the SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody and CHO-GD3 chimeric

antibody in that order. No difference in the ADCC activity was found between the NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT) prepared using different media in the culturing. The above results show that the ADCC activity of antibodies greatly varies depending on the kind of the animal cells to be used in their production. As its mechanism, since their antigen binding activities were identical, it was considered that it is caused by a difference in the structure binding to the antibody Fc region.

(0199)

### Example 3

Preparation of anti-human interleukin 5 receptor  $\alpha$  chain human CDR-grafted antibody:

1. Preparation of cell stably producing anti-human interleukin 5 receptor  $\alpha$  chain human CDR-grafted antibody

(1) Preparation of antibody-producing cell using rat myeloma YB2/0 cell

Using the anti-human interleukin 5 receptor  $\alpha$  chain human CDR-grafted antibody (hereinafter referred to as "anti-hIL-5R $\alpha$  CDR-grafted antibody") expression vector, pKANTEX1259HV3LV0, described in WO 97/10354, cells capable of stably producing anti-hIL-5R $\alpha$  CDR-grafted antibody were prepared as described below.

After introducing 5  $\mu$ g of the anti-hIL-5R $\alpha$  CDR-grafted antibody expression vector pKANTEX1259HV3LV0 into  $4 \times 10^6$  cells of rat myeloma YB2/0 by electroporation [Cytotechnology, 3, 133 (1990)], the cells were suspended in 40 ml of RPMI1640-FBS(10) and dispensed in 200  $\mu$ l/well into a 96 well culture plate (manufactured by Sumitomo Bakelite). After culturing them at 37°C for 24 hours in a 5% CO<sub>2</sub> incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from wells in which colonies of transformants showing G418 resistance were

formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5R $\alpha$  CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

(0200)

Regarding the transformants in wells in which production of the anti-hIL-5R $\alpha$  CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the them was suspended in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 50 nM MTX to give a density of 1 to  $2 \times 10^5$  cells/ml, and the suspension was dispensed in 2 ml into wells of a 24 well plate (manufactured by Greiner). Transformants showing 50 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO<sub>2</sub> incubator. The antigen binding activity of the anti-hIL-5R $\alpha$  CDR-grafted antibody in culture supernatants in wells in which growth of transformants was observed was measured by the ELISA shown in the item 2 of Example 3. Regarding the transformants in wells in which production of the anti-hIL-5R $\alpha$  CDR-grafted antibody was observed in culture supernatants, the MTX concentration was increased to 100 nM and then to 200 nM, and transformants capable of growing in the RPMI1640-FBS(10) medium comprising 0.5 mg/ml G418 and 200 nM MTX and of producing the anti-hIL-5R $\alpha$  CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line. The obtained anti-hIL-5R $\alpha$  CDR-grafted antibody-producing

transformed cell clone No. 3 has been deposited on April 5, 1999, as FERM BP-6690 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba, Ibaraki, Japan).

(0201)

(2) Preparation of antibody-producing cell using CHO/dhfr<sup>-</sup> cell

After introducing 4 µg of the anti-hIL-5Rα CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354 into  $1.6 \times 10^6$  cells of CHO/dhfr<sup>-</sup> by electroporation [Cytotechnology, 3, 133 (1990)], the cells were suspended in 10 ml of IMDM-FBS(10) and dispensed in 200 µl/well into a 96 well culture plate (manufactured by Iwaki Glass). After culturing them at 37°C for 24 hours in a 5% CO<sub>2</sub> incubator, G418 was added to give a concentration of 0.5 mg/ml, followed by culturing for 1 to 2 weeks. The culture supernatant was recovered from respective well in which colonies of transformants showing G418 resistance were formed and growth of colonies was observed, and the antigen binding activity of the anti-hIL-5Rα CDR-grafted antibody in the supernatant was measured by the ELISA shown in the item 2 of Example 3.

(0202)

Regarding the transformants in wells in which production of the anti-hIL-5Rα CDR-grafted antibody was observed in culture supernatants, in order to increase amount of the antibody production using a DHFR gene amplification system, each of the transformants was suspended in an IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 10 nM MTX to give a density of 1 to  $2 \times 10^5$  cells/ml, and the suspension was dispensed in 0.5 ml into wells of a 24 well plate (manufactured by Iwaki Glass). Transformants showing 10 nM MTX resistance were induced by culturing at 37°C for 1 to 2 weeks in a 5% CO<sub>2</sub> incubator. Regarding the transformants in wells in which their growth

was observed, the MTX concentration was increased to 100 nM and then to 500 nM, and transformants capable of growing in the IMDM-dFBS(10) medium comprising 0.5 mg/ml G418 and 500 nM MTX and of producing the anti-hIL-5R $\alpha$  CDR-grafted antibody in a large amount were finally obtained in the same manner as described above. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription product of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0203)

(3) Production of antibody-producing cell using mouse myeloma NS0 cell

An anti-hIL-5R $\alpha$  CDR-grafted antibody expression vector was prepared in accordance with the method of Yarranton et al. [*BIO/TECHNOLOGY*, 10, 169 (1992)] and using the antibody H chain cDNA and L chain cDNA on the anti-hIL-5R $\alpha$  CDR-grafted antibody expression vector pKANTEX1259HV3LV0 described in WO 97/10354, and NS0 cell was transformed to obtain transformants capable of producing the anti-hIL-5R $\alpha$  CDR-grafted antibody in a large amount. Among the obtained transformants, elite cell lines were selected and were made into a single cell (cloning) by limiting dilution twice. Also, using the method for determining the transcription production of an  $\alpha$ -1,6-fucosyltransferase gene shown in Example 9, a cell line producing a relatively small amount of the transcription product was selected and used as a suitable cell line.

(0204)

## 2. Measurement of binding activity of antibody to hIL-5R $\alpha$ (ELISA)

The binding activity of the antibody to hIL-5R $\alpha$  was measured as described below.

A solution was prepared by diluting the anti-hIL-5R $\alpha$  mouse antibody KM1257 described in WO 97/10354 with PBS to give a concentration of 10  $\mu$ g/ml, and 50  $\mu$ l of the resulting solution was dispensed into each well of a 96 well plate for ELISA (manufactured by Greiner), followed by reaction at 4°C for 20 hours. After the reaction, 1% BSA-PBS was dispensed in 100  $\mu$ l/well, and then the reaction was carried out at room temperature for 1 hour to block remaining active groups. After discarding 1% BSA-PBS, a solution prepared by diluting the soluble hIL-5R $\alpha$  described in WO 97/10354 with 1% BSA-PBS to give a concentration of 0.5  $\mu$ g/ml was dispensed in 50  $\mu$ l/well, followed by reaction at 4°C for 20 hours. After the reaction, each well was washed with Tween-PBS, culture supernatants of transformants or diluted solutions of a purified human CDR-grafted antibodies were dispensed in 50  $\mu$ g/well to carry out the reaction at room temperature for 2 hours. After the reaction, each well was washed with Tween-PBS, a peroxidase-labeled goat anti-human IgG (H & L) antibody solution (manufactured by American Qualex) diluted 3,000 times with 1% BSA-PBS was dispensed in 50  $\mu$ l/well as a secondary antibody solution, followed by reaction at room temperature for 1 hour. After the reaction and subsequent washing with Tween-PBS, the ABTS substrate solution was dispensed in 50  $\mu$ l/well for color development, and then the absorbance at OD415 was measured.

(0205)

## 3. Purification of anti-hIL-5R $\alpha$ CDR-grafted antibody

(1) Culturing of antibody-producing cell derived from YB2/0 cell and purification of antibody

The anti-hIL-5R $\alpha$  CDR-grafted antibody-producing transformed cell clone obtained in the item 1(1) of Example 3 was suspended in the GIT medium comprising 0.5 mg/ml G418 and 200 nM MTX to give a density of  $3 \times 10^5$  cells/ml and dispensed in 200 ml into 175 mm<sup>2</sup> flasks (manufactured by Greiner). After culturing them at 37°C for 8 days in a 5% CO<sub>2</sub> incubator, the culture supernatant was recovered. The anti-hIL-5R $\alpha$  CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5R $\alpha$  CDR-grafted antibody was named YB2/0-hIL-5R CDR antibody.

(2) Culturing of antibody-producing cell derived from CHO/dhfr<sup>-</sup> cell and purification of antibody

The anti-hIL-5R $\alpha$  CDR-grafted antibody-producing transformed cell clone obtained in the item 1(2) of Example 3 was suspended in the EX-CELL302 medium comprising 3 mM L-Gln, 0.5% CDLC and 0.3% PF68 to give a density of  $3 \times 10^5$  cells/ml and cultured using a 4.0 liter capacity spinner bottle (manufactured by Iwaki Glass) under agitating at a rate of 100 rpm. After culturing them at 37°C for 10 days in a temperature-controlling room, the culture supernatant was recovered. The anti-hIL-5R $\alpha$  CDR-grafted antibody was purified from the culture supernatant using ion exchange chromatography and a gel filtration method. The purified anti-hIL-5R $\alpha$  CDR-grafted antibody was named CHO/d-hIL-5R CDR antibody.

(0206)

(3) Culturing of antibody-producing cell derived from NS0 cell and purification of antibody

The anti-hIL-5R $\alpha$  CDR-grafted antibody-producing transformed cell clone obtained in the item 1(3) of Example 3 was cultured in accordance with the method of Yarranton et al. [BIO/TECHNOLOGY, 10, 169 (1992)] and then a culture supernatant was recovered. The anti-hIL-5R $\alpha$  CDR-grafted antibody was purified from the culture supernatant using

ion exchange chromatography and the gel filtration method. The purified anti-hIL-5R $\alpha$  CDR-grafted antibody was named NS0-hIL-5R CDR antibody.

(0207)

#### 4. Analysis of purified anti-hIL-5R $\alpha$ CDR-grafted antibodies

In accordance with a known method [*Nature*, 227, 680 (1970)], 4  $\mu$ g of each of the three kinds of the anti-hIL-5R $\alpha$  CDR-grafted antibodies produced by and purified from each animal cells, obtained in the item 3 of Example 3, was subjected to SDS-PAGE to analyze the molecular weight and purification degree. The results are shown in Fig. 4. As shown in Fig. 4, a single band of about 150 Kd in molecular weight was found under non-reducing conditions, and two bands of about 50 Kd and about 25 Kd under reducing conditions, in each of the purified anti-hIL-5R $\alpha$  CDR-grafted antibodies. The molecular weights almost coincided with the molecular weights deduced from the cDNA nucleotide sequences of H chain and L chain of the antibody (H chain: about 49 Kd, L chain: about 23 Kd, whole molecule: about 144 Kd), and also coincided with the reports stating that the IgG antibody has a molecular weight of about 150 Kd under non-reducing conditions and is degraded into H chains having a molecular weight of about 50 Kd and L chains having a molecular weight of about 25 Kd under reducing conditions due to cutting of the S-S bond in the molecule [*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 14 (1998); *Monoclonal Antibodies: Principles and Practice*, Academic Press Limited (1996)], so that it was confirmed that each anti-hIL-5R $\alpha$  CDR-grafted antibody was expressed and purified as an antibody molecule having the true structure.

(0208)

#### Example 4

Activity evaluation of anti-hIL-5R $\alpha$  CDR-grafted antibody:

1. Binding activity of anti-hIL-5R $\alpha$  CDR-grafted antibody to hIL-5R $\alpha$  (ELISA)

The activity of the three kinds of the purified anti-hIL-5R $\alpha$  CDR-grafted antibodies obtained in the item 3 of Example 3 to bind to hIL-5R $\alpha$  was measured by the ELISA shown in the item 2 of Example 3. Fig. 5 shows a result of the examination of the binding activity measured by changing concentration of the anti-hIL-5R $\alpha$  CDR-grafted antibody to be added. As shown in Fig. 5, the three kinds of the anti-hIL-5R $\alpha$  CDR-grafted antibodies showed almost the same binding activity to hIL-5R $\alpha$ . The result shows that the antigen binding activities of these antibodies are constant independently of the antibody-producing animal cells and their culturing methods, similar to the result of the item 1 of Example 2.

(0209)

2. *In vitro* cytotoxic activity (ADCC activity) of anti-hIL-5R $\alpha$  CDR-grafted antibody

In order to evaluate *in vitro* cytotoxic activity of the three kinds of the purified anti-hIL-5R $\alpha$  CDR-grafted antibodies obtained in the item 3 of Example 3, the ADCC activity was measured in accordance with the following method.

(1) Preparation of target cell solution

A mouse T cell line CTLL-2(h5R) expressing the hIL-5R $\alpha$  chain and  $\beta$  chain described in WO 97/10354 was cultured using the RPMI1640-FBS(10) medium to give a density of  $1 \times 10^6$  cells/0.5 ml, and the cells were radioisotope-labeled by reacting them with 3.7 MBq equivalents of a radioactive substance  $\text{Na}_2^{51}\text{CrO}_4$  at 37°C for 1.5 hours. After the reaction, the cells were washed three times through their suspension in the RPMI1640-FBS(10) medium and centrifugation, resuspended in the medium and then incubated at 4°C for 30 minutes in ice for spontaneous dissolution of the radioactive substance. After the

centrifugation, the precipitate was adjusted to give a density of  $2 \times 10^5$  cells/ml by adding 5 ml of the RPMI1640-FBS(10) medium and used as the target cell solution.

(0210)

(2) Preparation of effector cell solution

From a healthy person, 50 ml of venous blood was collected and gently mixed with 0.5 ml of heparin sodium (manufactured by Takeda Pharmaceutical). The mixture was centrifuged to separate a mononuclear cell layer using Polymorphprep (manufactured by Nycomed Pharma AS) and in accordance with the manufacture's instructions. After washing with the RPMI1640-FBS(10) medium by centrifugation three times, the resulting cells were resuspended to give a density of  $9 \times 10^6$  cells/ml using the medium and used as the effector cell solution.

(0211)

(3) Measurement of ADCC activity

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50  $\mu$ l of the target cell solution prepared in the above (1) ( $1 \times 10^4$  cells/well) was dispensed. Next, 100  $\mu$ l of the effector cell solution prepared in the above (2) was dispensed ( $9 \times 10^5$  cells/well, the ratio of effector cells to target cells becomes 90:1). Subsequently, each of the anti-hIL-5R $\alpha$  CDR-grafted antibodies was added to give a final concentration of 0.001 to 0.1  $\mu$ g/ml, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, and the amount of  $^{51}\text{Cr}$  in the supernatant was measured using a  $\gamma$ -counter. The amount of spontaneously released  $^{51}\text{Cr}$  was calculated by the same operation using only the medium instead of the effector cell solution and the antibody solution, and measuring the amount of  $^{51}\text{Cr}$  in the supernatant. The amount of total released  $^{51}\text{Cr}$  was calculated by the same operation using only the medium instead of the antibody solution and adding 1 N

hydrochloric acid instead of the effector cell solution, and measuring the amount of  $^{51}\text{Cr}$  in the supernatant. The ADCC activity was calculated from the above equation (I).

(0212)

The results are shown in Fig. 6. As shown in Fig. 6, among the three kinds of the anti-hIL-5R $\alpha$  CDR-grafted antibodies, the YB2/0-hIL-5R CDR antibody showed the most potent ADCC activity, followed by the CHO/d-hIL-5R CDR antibody and the NS0-hIL-5R CDR antibody in this order. Similar to the result of the item 2 of Example 2, the above results show that the ADCC activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since the antibodies produced by the YB2/0 cell showed the most potent ADCC activity in both cases of the two kinds of the humanized antibodies, it was revealed that an antibody having potent ADCC activity can be produced by using the YB2/0 cell.

(0213)

### 3. *In vivo* activity evaluation of anti-hIL-5R $\alpha$ CDR-grafted antibody

In order to evaluate *in vivo* activity of the three kinds of the purified anti-hIL-5R $\alpha$  CDR-grafted antibodies obtained in the item 3 of Example 3, the inhibition activity in an hIL-5-induced eosinophilia increasing model of *Macaca fascicularis* was examined in accordance with the following method.

The hIL-5 (preparation method is described in WO 97/10354) was administered to *Macaca fascicularis* under the dorsal skin at a dose of 1  $\mu\text{g}/\text{kg}$ , starting on the first day and once a day for a total of 14 times. Each anti-hIL-5R $\alpha$  CDR-grafted antibody was intravenously administered at a dose of 0.3 mg/kg one hour before the hIL-5 administration on the day zero. An antibody-non-added group was used as the control. In the antibody-administered groups, three animals of *Macaca fascicularis* were used in each group

(No. 301, No. 302, No. 303, No. 401, No. 402, No. 403, No. 501, No. 502 and No. 503), and two animals (No. 101 and No. 102) were used in the antibody-non-added group. Starting 7 days before commencement of the administration and until 42 days after the administration, about 1 ml of blood was periodically collected from a saphena or a femoral vein, and the number of eosinophils in 1  $\mu$ l of peripheral blood was measured. The results are shown in Fig. 7. As shown in Fig. 7, increase in the blood eosinophil was completely inhibited in the group to which the YB2/0-hIL-5R CDR antibody was administered. On the other hand, complete inhibition activity was found in one animal in the group to which the CHO/d-hIL-5R CDR antibody was administered, but the inhibition activity was not sufficient in two animals. In the group to which NS0-hIL-5R CDR antibody was administered, complete inhibition activity was not found and its effect was not sufficient.

(0214)

The above results show that the *in vivo* activity of antibodies greatly varies depending on the animal cells to be used in their production. In addition, since a positive correlation was found between the degree of the *in vivo* activity of the anti-hIL-5R $\alpha$  CDR-grafted antibody and the degree of its ADCC activity described in the item 2 of Example 4, it was indicated that the degree of ADCC activity is remarkably important for its activity expression.

Based on the above results, it is expected that an antibody having potent ADCC activity is useful also in the clinical field for various diseases in human.

(0215)

#### Example 5

Analysis of sugar chain which enhances ADCC activity:

1. Preparation of 2-aminopyridine-labeled sugar chain (PA-treated sugar chain)

The humanized antibody of the present invention was acid-hydrolyzed with hydrochloric acid to remove sialic acid. After hydrochloric acid was completely removed, the sugar chain was cleaved from the protein by hydrazinolysis [*Method of Enzymology*, 83, 263 (1982)]. Hydrazine was removed, and N-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After lyophilizing, fluorescence labeling with 2-aminopyridine was carried out [*J. Biochem.*, 95, 197 (1984)]. The fluorescence-labeled sugar chain (PA-treated sugar chain) was separated from impurity using Surperdex Peptide HR 10/30 Column (manufactured by Pharmacia). The sugar chain fraction was dried using a centrifugal concentrator and used as a purified PA-treated sugar chain. (0216)

## 2. Reverse phase HPLC analysis of PA-treated sugar chain of purified anti-hIL-5R $\alpha$ CDR-grafted antibody

According to the method in the item 1 of Example 5, various anti-hIL-5R $\alpha$  CDR-grafted antibodies produced in Example 3 were subjected to PA-treated sugar chain treatment, and reverse phase HPLC analysis was carried out by CLC-ODS column (manufactured by Shimadzu). An excess amount of  $\alpha$ -L-fucosidase (derived from bovine kidney, manufactured by SIGMA) was added to the PA-treated sugar chain for digestion (37°C, 15 hours), and then the products were analyzed by reverse phase HPLC (Fig. 8). It was confirmed that the asparagine-linked sugar chain is eluted for 30 minutes to 80 minute using PA-treated sugar chain standards manufactured by Takara Shuzo. The ratio of sugar chains whose reverse phase HPLC elution positions were shifted (sugar chains eluted for 48 minutes to 78 minutes) by the  $\alpha$ -L-fucosidase digestion was calculated. The results are shown in Table 1.

(0217)

(Table 1)

Table 1

Antibody-producing cell	$\alpha$ -1,6-Fucose-linked sugar chain (%)
YB2/0	47
NS0	73

(0218)

About 47% of the anti-hIL-5R CDR-grafted antibody produced by the YB2/0 cell and about 73% of the anti-hIL-5R CDR-grafted antibody produced by the NS0 cell were sugar chains in which 1-position of fucose is bound to 6-position of *N*-acetylglucosamine in the reducing end through  $\alpha$ -bond in the *N*-glycoside-linked sugar chain (hereinafter referred to as "sugar chain having  $\alpha$ -1,6-fucose"). Thus, the ratio of sugar chains in which 1-position of fucose is not bound to 6-position of *N*-acetylglucosamine in the reducing end through  $\alpha$ -bond in the *N*-glycoside-linked sugar chain (hereinafter referred to as " $\alpha$ -1,6-fucose-free sugar chain") is higher in the antibody produced by the YB2/0 cell than in the antibody produced by the NS0 cell.

(0219)

### 3. Analysis of monosaccharide composition of purified anti-hIL-5R $\alpha$ CDR-grafted antibody

Sugar chains of anti-hIL-5R $\alpha$  CDR-grafted antibodies produced by the YB2/0 cell, NS0 cell and CHO/d cell were hydrolyzed into monosaccharides by acid hydrolysis with trifluoroacetic acid, and monosaccharide composition analysis was carried out using BioLC (manufactured by Dionex).

Among *N*-glycoside-linked sugar chains, there are 3 mannose units in one sugar chain in the complex type *N*-glycoside-linked sugar chain. A relative ratio of each monosaccharide obtained by calculating the number of mannose as 3 is shown in Table 2.

(0220)

(Table 3)

Table 2

Antibody-producing cell	Fuc	GlcNAc	Gal	Man	ADCC activity (%)*
YB2/0	0.60	4.98	0.30	3.00	42.27
NS0	1.06	3.94	0.66	3.00	16.22
CHO/dhFr <sup>-</sup>	0.85	3.59	0.49	3.00	25.73
CHO/dhFr <sup>-</sup>	0.91	3.80	0.27	3.00	25.73

\*: Antibody concentration: 0.01 µg/ml

(0221)

Since the relative ratios of fucose were in an order of YB2/0 < CHO/d < NS0, the sugar chain produced in the antibody produced by YB2/0 cell showed the lowest fucose content as also shown in the present results.

(0222)

Example 6

Sugar chain analysis of antibody produced by CHO/dhfr<sup>-</sup> cell:

PA-treated sugar chains were prepared from purified anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr<sup>-</sup> cell, and reverse phase HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 9). In Fig. 9, an elution time from 35 to 45 minutes corresponded to sugar chains having no fucose and an elution time from 45 to 60 minutes corresponded to sugar chains having fucose. Similar to the case of the antibody produced by mouse myeloma NS0 cell, the anti-hIL-5Rα CDR-grafted antibody produced by CHO/dhfr<sup>-</sup> cell had less fucose-free sugar chain content than the antibody produced by rat myeloma YB2/0 cell.

(0223)

Example 7

Separation of potent ADCC activity antibody:

The anti-hIL-5Rα CDR-grafted antibody produced by rat myeloma YB2/0 cell was separated using a lectin column which binds to sugar chains having fucose. HPLC was

carried out using LC-6A manufactured by Shimadzu at a flow rate of 1 ml/min and at room temperature as the column temperature. After equilibration with 50 mM Tris-sulfate buffer (pH 7.3), the purified anti-hIL-5R $\alpha$  CDR-grafted antibody was injected and then eluted by a linear density gradient (60 minutes) of 0.2 M  $\alpha$ -methylmannoside (manufactured by Nakalai Tesque). The anti-hIL-5R $\alpha$  CDR-grafted antibody was separated into non-adsorbed fraction and adsorbed fraction. When the non-adsorbed fraction and a part of the adsorbed fraction were sampled and their binding activity to hIL-5R $\alpha$  was measured, they showed similar binding activity (Fig. 10 upper). When the ADCC activity was measured, the non-adsorbed fraction showed potent ADCC activity (100 to 1000 folds) than that of the part of adsorbed fraction (Fig. 10 lower). In addition, PA-treated sugar chains were prepared from the non-adsorbed fraction and a part of the adsorbed fraction, and reverse HPLC analysis was carried out using CLC-ODS column (manufactured by Shimadzu) (Fig. 11). In the non-adsorbed fraction, an antibody binding to fucose-free sugar chains was mainly present, and in the part of adsorbed fraction, an antibody binding to sugar chains having fucose was mainly present.

(0224)

#### Example 8

Determination of transcription product of  $\alpha$ -1,6-fucosyltransferase (FUT8) gene in host cell line:

(1) Preparation of single-stranded cDNA from various cell lines

Single-stranded cDNA samples were prepared from dihydrofolate reductase gene (dhfr)-deleted CHO/DG44 cells derived from Chinese hamster ovary and rat myeloma YB2/0 cells by the following procedure.

The CHO/DG44 cells were suspended in IMDM medium (manufactured by Life Technologies) supplemented with 10%

fetal bovine serum (manufactured by Life Technologies) and 1 x concentration HT supplement (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for adhesion cell culture use (manufactured by Greiner) at a density of  $2 \times 10^5$  cells/ml. Also, the YB2/0 cells were suspended in RPMI 1640 medium (manufactured by Life Technologies) supplemented with 10% fetal bovine serum (manufactured by Life Technologies) and 4 mmol/l L-GLN (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of  $2 \times 10^5$  cells/ml. They were cultured at 37°C in a 5% CO<sub>2</sub> incubator, and  $1 \times 10^7$  of respective host cells were recovered on the 1st, 2nd, 3rd, 4th and 5th days of the culturing to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

(0225)

The total RNA was dissolved in 45 µl of sterile water, 1 µl of RQ1 RNase-Free DNase (manufactured by Promega), 5 µl of the attached 10 x DNase buffer and 0.5 µl of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 µl of sterile water.

In a 20 µl of the reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 µg of each of the obtained total RNA samples by reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) and in accordance with the manufacture's instructions. A 1 x concentration solution of the reaction solution was used for the cloning of α-

1,6-fucosyltransferase (hereinafter referred sometimes to as "FUT8") and  $\beta$ -actin derived from respective host cells, and 50 folds-diluted aqueous solution of the reaction solution for the determination of each gene transcription amount by competitive PCR, and the solutions were stored at -80°C until use.

(0226)

(2) Preparation of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8

Each cDNA partial fragment of Chinese hamster FUT8 and rat FUT8 was prepared by the following procedure (Fig. 12).

First, primers (shown in SEQ ID NOs:4 and 5) specific for nucleotide sequences common to human FUT8 cDNA [*J. Biochem.*, 121, 626 (1997)] and swine FUT8 cDNA [*J. Biol. Chem.*, 271, 27810 (1995)] were designed.

Next, 25  $\mu$ l of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs and 0.5  $\mu$ mol/l gene-specific primers (SEQ ID NOs:4 and 5)] containing 1  $\mu$ l of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing, and polymerase chain reaction (PCR) was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0227)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 979 bp was purified using GENECLAN Spin Kit (manufactured by BIO 101) and eluted with 10  $\mu$ l of sterile water (hereinafter, the method was used for the purification of DNA fragments from agarose gel). Into a

plasmid pCR2.1, 4  $\mu$ l of the amplified fragment was employed to insert in accordance with the manufacture's instructions of TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* XL1-Blue was transformed with the reaction solution by the method of Cohen et al. [*Proc. Natl. Acad. Sci. USA*, **69**, 2110 (1972)] (hereinafter, the method was used for the transformation of *E. coli*). Plasmid DNA samples were isolated in accordance with a known method [*Nucleic Acids Research*, **7**, 1513 (1979)] (hereinafter, the method was used for the isolation of plasmid) from cDNA-inserted 6 clones among the obtained kanamycin-resistant colonies.

(0228)

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the open reading frame (ORF) partial sequences of Chinese hamster FUT8 or rat FUT8 (shown in SEQ ID NOs:6 and 7). Among these, plasmid DNA samples containing absolutely no reading error by the PCR in the sequences were selected. Herein, these plasmids are referred to as CHFUT8-pCR2.1 and YBFUT8-pCR2.1.

(0229)

(3) Preparation of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin cDNA

Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin cDNA were prepared by the following procedure (Fig. 13).

First, a forward primer specific for a common sequence containing translation initiation codon (shown in SEQ ID NO:8) and reverse primers specific for respective sequences containing translation termination codon (shown in SEQ ID NOs:9 and 10) were designed from Chinese hamster

$\beta$ -actin genomic sequence (GenBank, U20114) and rat  $\beta$ -actin genomic sequence [*Nucleic Acids Research*, 11, 1759 (1983). (0230)

Next, 25  $\mu$ l of a reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l  $MgCl_2$ , 0.4  $\mu$ mol/l gene-specific primers (SEQ ID NOs:8 and 9, or SEQ ID NOs:8 and 10) and 5% DMSO] containing 1  $\mu$ l of each of the cDNA prepared from CHO cell and cDNA prepared from YB2/0 cell, both obtained in the item (1) 2 days after culturing was prepared, and PCR was carried out using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94°C for 1 minute and subsequent 25 cycles of heating at 98°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds as one cycle. (0231)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1128 bp was purified. The DNA fragment was subjected to DNA 5'-terminal phosphorylation using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions. The DNA fragment was recovered from the reaction solution using ethanol precipitation method and dissolved in 10  $\mu$ l of sterile water.

Separately, 3  $\mu$ g of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 16 units of a restriction enzyme *EcoRV* (manufactured by Takara Shuzo) were added thereto for digestion reaction at 37°C for 3 hours. To the reaction solution, 35  $\mu$ l of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5  $\mu$ l of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to thereby dephosphorylate the DNA terminus. The reaction solution was extracted with phenol/chloroform, followed by

ethanol precipitation, and the recovered DNA fragment was dissolved in 100  $\mu$ l of sterile water.

(0232)

Each 4  $\mu$ l of the amplified fragment prepared from Chinese hamster cDNA or the amplified fragment (1192 bp) prepared from rat cDNA was mixed with 1  $\mu$ l of the *EcoRV*-*EcoRV* fragment (about 3.0 Kb) prepared from plasmid pBluescript II KS(+) and 5  $\mu$ l of Ligation High (manufactured by Toyobo) for ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* XL1-Blue was transformed, and plasmid DNA samples were isolated respectively in accordance with a known method from the obtained ampicillin-resistant clones.

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined by the method encode the ORF full sequences of Chinese hamster  $\beta$ -actin or rat  $\beta$ -actin. Among these, plasmid DNA samples containing absolutely no reading error of bases by the PCR in the sequences were selected. Herein, the plasmids are called CHAc-pBS and YBAC-pBS.

(0233)

#### (4) Preparation of FUT8 standard and internal control

In order to measure a transcription level of FUT8 gene mRNA in each cell, CHFT8-pCR2.1 or YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in the item (2) from Chinese hamster FUT8 or rat FUT8 were inserted into pCR2.1, respectively, were digested with a restriction enzyme *EcoRI*, and the obtained linear DNAs were used as the standards for the preparation of a calibration curve. CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were

obtained from the CHFT8-pCR2.1 and YBFT8-pCR2.1, by deleting 203 bp between *ScaI* and *HindIII*, an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, were digested with a restriction enzyme *EcoRI*, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination. Details thereof are described below.

(0234)

Chinese hamster FUT8 and rat FUT8 standards were prepared as follows. In 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), 2  $\mu$ g of the plasmid CHFT8-pCR2.1 was dissolved, 24 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2  $\mu$ g of the plasmid YBFT8-pCR2.1 was dissolved in 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. By subjecting a portion of each of the reaction solutions to 0.8% agarose gel electrophoresis, it was confirmed that an *EcoRI*-*EcoRI* fragment (about 1 Kb) containing each of cDNA partial fragments of Chinese hamster FUT8 and rat FUT8 was separated from the plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1 by the restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1  $\mu$ g/ml of baker's yeast t-RNA (manufactured by SIGMA) to give a concentration of 0.02 fg/ $\mu$ l, 0.2 fg/ $\mu$ l, 1 fg/ $\mu$ l, 2 fg/ $\mu$ l, 10 fg/ $\mu$ l, 20 fg/ $\mu$ l and 100 fg/ $\mu$ l and used as the Chinese hamster FUT8 and rat FUT8 standards.

(0235)

Internal standards of Chinese hamster FUT8 and rat FUT8 were prepared as follows (Fig. 14). A reaction solution [KOD buffer #1 (manufactured by Toyobo), 0.2 mmol/l dNTPs, 1 mmol/l  $MgCl_2$ , 0.4  $\mu$ mol/l gene-specific primers (SEQ ID NOs:11 and 12) and 5% DMSO] containing 5 ng

of CHFT8-pCR2.1 or YBFT8-pCR2.1 was prepared, and PCR was carried out using a DNA polymerase KOD (manufactured by Toyobo). The PCR was carried out by heating at 94°C for 4 minutes and subsequent 25 cycles of heating at 98°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds as one cycle. After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of about 4.7 Kb was purified. The DNA 5'-terminal was phosphorylated using MEGALABEL (manufactured by Takara Shuzo) in accordance with the manufacture's instructions, and then the DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 50 µl of sterile water. The obtained DNA fragment (5 µl, about 4.7 kb) and 5 µl of Ligation High (manufactured by Toyobo) were mixed, followed by self-cyclization reaction at 16°C for 30 minutes.

(0236)

Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNA samples were isolated in accordance with a known method from the obtained ampicillin-resistant clones. The nucleotide sequence of each plasmid DNA was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a 203 bp inner nucleotide sequence between *ScaI* and *HindIII* of Chinese hamster FUT8 or rat FUT8 was deleted. The obtained plasmids are referred to as CHFT8d-pCR2.1 or YBFT8d-pCR2.1, respectively.

(0237)

Next, 2 µg of the plasmid CHFT8d-pCR2.1 was dissolved in 40 µl of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2 µg of the plasmid YBFT8d-pCR2.1 was dissolved

in 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 24 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *EcoRI-EcoRI* fragment (about 800 bp) containing a fragment from which 203 bp of the inner nucleotide sequences of Chinese hamster FUT8 or rat FUT8 partial fragments was deleted was separated from the plasmids CHFT8d-pCR2.1 or YBFT8d-pCR2.1 by the restriction enzyme digestion reactions. Dilutions of 2 fg/ $\mu$ l were prepared from the reaction solutions using 1  $\mu$ g/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster FUT8 or rat FUT8 internal controls.

(0238)

(5) Preparation of  $\beta$ -actin standard and internal control

In order to measure the transcription amount of  $\beta$ -actin gene mRNA in various host cells, CHAc-pBS and YBAC-pBS, as plasmids in which the ORF full length of each cDNA of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin prepared in the item (3) was inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *HindIII* and *PstI* and restriction enzymes *HindIII* and *KpnI*, respectively, and the digested linear DNAs were used as the standards for the preparation of a calibration curve. CHAc-d-pBS and YBAC-d-pBS which were obtained from the CHAc-pBS and YBAC-pBS by deleting 180 bp between *DraIII* and *DraIII* of an inner nucleotide sequence of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin were digested with restriction enzymes *HindIII* and *PstI* and restriction enzymes *HindIII* and *KpnI*, respectively, and the digested linear DNAs were used as the internal standards for  $\beta$ -actin amount determination. Details thereof are described below.

(0239)

Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin standards were prepared as follows. In 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), 2  $\mu$ g of the plasmid CHAc-pBS was dissolved, and 25 units of a restriction enzyme *Hind*III (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2  $\mu$ g of the plasmid YBAC-pBS was dissolved in 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hind*III (manufactured by Takara Shuzo) and 25 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that a *Hind*III-*Pst*I fragment and a *Hind*III-*Kpn*I fragment (about 1.2 Kb) containing the full length ORF of each cDNA of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin were separated from the plasmids CHAc-pBS and YBAC-pBS by the restriction enzyme digestion reactions. Each of the reaction solutions was diluted with 1  $\mu$ g/ml baker's yeast t-RNA (manufactured by SIGMA) to give a concentration 2 pg/ $\mu$ l, 1 pg/ $\mu$ l, 200 fg/ $\mu$ l, 100 fg/ $\mu$ l and 20 fg/ $\mu$ l and used as the Chinese hamster  $\beta$ -actin and or  $\beta$ -actin standards.

(0240)

Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin internal standards were prepared as follows (Fig. 15). In 100  $\mu$ l of NEBuffer 3 (manufactured by New England Biolabs) containing 100 ng/ $\mu$ l of BSA (manufactured by New England Biolabs), 2  $\mu$ g of CHAc-pBS was dissolved, and 10 units of a restriction enzyme *Dra*III (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 3 hours. DNA fragments were recovered from the reaction solution by ethanol precipitation and the DNA termini were changed to blunt ends using DNA Blunting Kit (manufactured

by Takara Shuzo) in accordance with the manufacture's instructions, and then the reaction solution was divided into two equal parts. First, to one part of the reaction solution, 35  $\mu$ l of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5  $\mu$ l of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes for dephosphorylating the DNA termini. The DNA fragment was recovered by carrying out dephosphorylation treatment, phenol/chloroform extraction treatment and ethanol precipitation treatment and then dissolved in 10  $\mu$ l of sterile water. The remaining part of the reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.1 Kb containing the ORF partial fragment of Chinese hamster  $\beta$ -actin.

(0241)

The dephosphorylated *Dra*III-*Dra*III fragment (4.5  $\mu$ l), 4.5  $\mu$ l of the *Dra*III-*Dra*III fragment of about 1.1 Kb and 5  $\mu$ l of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5 $\alpha$  was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. The nucleotide sequence of each plasmid DNA was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer), and it was confirmed that a Chinese hamster  $\beta$ -actin *Dra*III-*Dra*III 180 bp inserted into the plasmid was deleted. The plasmid is referred to as CHAc-d-pBS.

(0242)

Also, a plasmid in which rat  $\beta$ -actin *Dra*III-*Dra*III 180 bp was deleted was prepared via the same steps of CHAc-d-pBS. The plasmid is referred to as YBAc-d-pBS.

Next, 2  $\mu$ g of the plasmid CHAc-d-pBS was dissolved in 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hind*III (manufactured by Takara Shuzo) and 20 units of *Pst*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. Separately, 2  $\mu$ g of the plasmid YBAc-d-pBS was dissolved in 40  $\mu$ l of NEBuffer 2 (manufactured by New England Biolabs), and 25 units of a restriction enzyme *Hind*III (manufactured by Takara Shuzo) and 24 units of *Kpn*I (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 3 hours. A portion of each of the reaction solutions was subjected to 0.8% agarose gel electrophoresis, and it was confirmed that an *Hind*III-*Pst*I fragment and *Hind*III-*Kpn*I fragment (about 1.0 Kb) containing a fragment in which 180 bp of the inner nucleotide sequence of the ORF full length of each cDNA of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin was deleted were separated from the plasmids CHAc-d-pBS and YBAc-d-pBS by the restriction enzyme digestion reactions. Dilutions of 200 fg/ $\mu$ l were prepared from the reaction solutions using 1  $\mu$ g/ml baker's yeast t-RNA (manufactured by SIGMA) and used as the Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin internal controls.

(0243)

(6) Determination of transcription amount by competitive PCR

Competitive PCR was carried out using the FUT8 internal control DNA prepared in the item (4) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the FUT8 transcription product in the host cell line was calculated from the relative value of the amount of the amplified product derived from each template. On the other hand, since it is considered that the  $\beta$ -actin gene is transcribed continuously in each cell and its transcription level is approximately the same

between cells, transcription level of the  $\beta$ -actin gene was determined as a measure of the efficiency of synthesis reaction of cDNA in each host cell line. That is, the PCR was carried out using the  $\beta$ -actin internal control DNA prepared in the item (5) and the host cell-derived cDNA obtained in the item (1) as the templates, the determined value of the  $\beta$ -actin transcription product in the host cell line was calculated from the relative value of the amount of the amplified product derived from each template. Details thereof are described below.

(0244)

The FUT8 transcription product was determined by the following procedure. First, a set of sequence-specific primers (shown in SEQ ID NOs:13 and 14) common to the inner sequences of the ORF partial sequences of Chinese hamster FUT8 and rat FUT8 obtained in the item (2) were designed. Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20  $\mu$ l in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from each of respective host cell line in the item (1) and 5  $\mu$ l (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0245)

Also, PCR was carried out in a series of reaction in which 5  $\mu$ l (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid obtained in the item (4) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription level.

The  $\beta$ -actin transcription product was determined by the following procedure. First, two sets of respective gene-specific primers common to the inner sequences of the ORF full lengths of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin obtained in the item (3) were designed (the former are shown in SEQ ID NOs:15 and 16, and the latter are shown in SEQ ID NOs:17 and 18).

Next, PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20  $\mu$ l in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l gene-specific primers (SEQ ID NOs:15 and 16, or SEQ ID NOs:17 and 18) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from respective host cell line in the item (1) and 5  $\mu$ l (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle.

(0246)

Also, PCR was carried out in a series of reaction in which 5  $\mu$ l (10 pg, 5 pg, 1 pg, 500 fg or 100 fg) of the  $\beta$ -actin standard plasmid obtained in the item (5) was added instead of the each host cell line-derived cDNA, and used in the preparation of a calibration curve for the  $\beta$ -actin transcription level.

(0247)

(Table 3)

Table 3

Target gene	Primer set *	Size (bp) of PCR amplification product	
		Target	Competitor
FUT8	F: 5'-GTCCATGGTGATCCTGCAGTGTGG-3' R: 5'-CACCAATGATATCTCCAGGTTCC-3'	638	431
$\beta$ -Actin (Chinese hamster)	F: 5'-GATATCGCTGCGCTCGTTGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAAAGAGC-3'	789	609
$\beta$ -Actin (Rat)	F: 5'-GATATCGCTGCGCTCGTCGTCGAC-3' R: 5'-CAGGAAGGAAGGCTGGAAGAGAGC-3'	789	609

\* F: forward primer, R: reverse primer

(0248)

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

A 7  $\mu$ l portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1  $\times$  concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

(0249)

The amount of an amplified product formed by PCR using a standard plasmid as the template was measured by the method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was

calculated from the amount of the amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

The amount of the FUT8 transcription product in each host cell line when a rat FUT8 sequence was used in the standard and internal control is shown in Fig. 16. Throughout the culturing period, the CHO cell line showed a transcription amount 10 folds or higher than that of the YB2/0 cell line. The tendency was also found when a Chinese hamster FUT8 sequence was used in the standard and internal control.

Also, the FUT8 transcription amounts are shown in Table 4 as relative values to the amount of the  $\beta$ -actin transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB2/0 cell line was around 0.1% of  $\beta$ -actin while it was 0.5% to 2% in the CHO cell line.

The results shows that the amount of the FUT8 transcription product in YB2/0 cell line was significantly smaller than that in the CHO cell line.

(0250)

(Table 4)

Table 4

Cell line	Culture days				
	1st	2nd	3rd	4th	5th
CHO	1.95	0.90	0.57	0.52	0.54
YB2/0	0.12	0.11	0.14	0.08	0.07

(0251)

Example 9

Determination of transcription product of  $\alpha$ -1,6-fucosyltransferase (FUT8) gene in anti-ganglioside GD3 chimeric antibody-producing cell line:

(1) Preparation of single-stranded cDNA from various antibody-producing cell lines

Single-stranded cDNA was prepared from anti-ganglioside GD3 chimeric antibody-producing cell lines DCHI01-20 and 61-33 as follows. The DCHI01-20 is a transformant clone derived from the CHO/DG44 cell described in item 2(2) of Example 1. Also, the 61-33 is a clone obtained by carrying out serum-free adaptation of YB2/0-derived transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan)) and then carrying out single cell isolation by two limiting dilution.

(0252)

Cells of the DCHI01-20 were suspended in EXCELL 302 medium (manufactured by JRH BIOSCIENCES) supplemented with 3 mmol/l L-GLN (manufactured by Life Technologies), 0.3% PLURONIC F-68 (manufactured by Life Technologies) and 0.5% fatty acid concentrate (manufactured by Life Technologies), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture use (manufactured by Greiner) at a density of  $2 \times 10^5$  cells/ml. Also, cells of the 61-33 were suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.2% bovine serum albumin fraction V (manufactured by Life Technologies) (hereinafter referred to as "BSA"), and 15 ml of the suspension was inoculated into T75 flask for suspension cell culture (manufactured by Greiner) at a density of  $2 \times 10^5$  cells/ml. They were cultured at 37°C in a 5% CO<sub>2</sub>,

incubator, and 1, 2, 3, 4 and 5 days after culturing,  $1 \times 10^7$  of respective host cells were recovered to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions.

(0253)

The total RNA was dissolved in 45  $\mu$ l of sterile water, and 1  $\mu$ l of RQ1 RNase-Free DNase (manufactured by Promega), 5  $\mu$ l of the attached 10  $\times$  DNase buffer and 0.5  $\mu$ l of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50  $\mu$ l of sterile water.

In a 20  $\mu$ l reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3  $\mu$ g of each of the obtained total RNA samples by reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. The reaction solution was diluted 50 folds with water and stored at -80°C until use.

(0254)

(2) Determination of transcription amounts of each gene by competitive PCR

The transcription amount of each of the genes on the cDNA derived from the antibody-producing cell line obtained in the item (1) was determined by competitive PCR in accordance with Example 9(6).

The FUT8 gene-derived mRNA transcription amount in each of the antibody-producing cell lines was determined by the following procedure.

CHFT8-pCR2.1 and YBFT8-pCR2.1, as plasmids in which cDNA partial fragments prepared in Example 9(2) from Chinese hamster FUT8 and rat FUT8, respectively, were

inserted into pCR2.1, were digested with a restriction enzyme *EcoRI*, and the obtained linear DNAs were used as the standards in the preparation of a calibration curve for determining the FUT8 transcription amount.

CHFT8d-pCR2.1 and YBFT8d-pCR2.1, which were obtained by deleting 203 bp between *ScaI* and *HindIII* of an inner nucleotide sequence of Chinese hamster FUT8 and rat FUT8, respectively, in Example 8(4) were digested with a restriction enzyme *EcoRI*, and the obtained linear DNAs were used as the internal standards for FUT8 amount determination.

(0255)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20  $\mu$ l in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from each of the antibody-producing cell line in the item (1) and 5  $\mu$ l (10 fg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0256)

Also, PCR was carried out in a series of reaction in which 5  $\mu$ l (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid was added instead of the each antibody-producing cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1  $\mu$ g/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of the standard plasmid.

On the other hand, since it is considered that the  $\beta$ -actin gene is transcribed constantly in each cell and its transcription amount is approximately the same between

cells, the transcription amount of the  $\beta$ -actin gene was determined as an index of the efficiency of synthesis reaction of cDNA in each antibody-producing cell line.

CHAc-pBS and YBAC-pBS as plasmids in which the ORF full length of each cDNA of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin prepared in Example 8(3) were inserted into pBluescript II KS(+), respectively, were digested with restriction enzymes *HindIII* and *KpnI*, and the obtained linear DNA samples were used as the standards in the preparation of a calibration curve for determining the  $\beta$ -actin transcription amount.

(0257)

CHAc-d-pBS and YBAC-d-pBS which were obtained by deleting 180 bp between *DraI* and *DraI* of an inner nucleotide sequence of Chinese hamster  $\beta$ -actin and rat  $\beta$ -actin, respectively in Example 8(5), were digested with restriction enzymes *HindIII* and *KpnI*, and the obtained linear DNAs were used as the internal standards for  $\beta$ -actin determination.

(0258)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20  $\mu$ l in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l  $\beta$ -actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from each of the antibody-producing cell lines and 5  $\mu$ l (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle. Also, PCR was carried out in a series of reaction in which 10 pg, 5 pg, 1 pg, 500 fg or 100 fg of the  $\beta$ -actin standard plasmid was added instead of the each antibody-producing cell line-derived cDNA, and used in the preparation of a calibration curve for the  $\beta$ -

actin transcription amount. In this case, 1 µg/ml of a baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

(0259)

By PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

A 7 µl portion of each of the solutions after PCR was subjected to 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1 x concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). The amount of the amplified DNA fragment was measured by calculating luminescence intensity of each amplified DNA using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics).

(0260)

The amount of the amplified product formed by PCR which used a standard plasmid as the template was measured by the method, and a calibration curve was prepared by plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was calculated from the amount of the amplified product when each antibody-producing cell line-derived cDNA was used as the template, and the value was defined as the mRNA transcription amount in each cell line.

(0261)

The FUT8 transcription amounts are shown in Table 5 as relative values to the amount of the β-actin transcription product. Throughout the culturing period, the FUT8 transcription amount in the YB2/0 cell-derived

antibody-producing 61-33 was 0.3% or less of  $\beta$ -actin while it was 0.7% to 1.5% in the CHO cell-derived antibody-producing cell. The results shows that the amount of the FUT8 transcription product in the YB2/0 cell-derived antibody-producing cell line was significantly less than that in the antibody-producing cell line derived from the CHO cell.

(0262)

(Table 5)

Table 5

Cell line	Culture days				
	1st	2nd	3rd	4th	5th
DCHI01-20	0.75	0.73	0.99	1.31	1.36
61-33	0.16	0.19	0.24	0.30	<0.10

(0263)

#### Example 10

Preparation of mouse  $\alpha$ -1,6-fucosyltransferase (FUT8) gene over-expressing cell line:

(1) Construction of mouse  $\alpha$ -1,6-fucosyltransferase (FUT8) expression plasmid

Total RNA was extracted from  $1 \times 10^7$  cells of a mouse myeloma NSO cell (RCB0213, Cell Bank at The Institute of Physical and Chemical Research) subcultured using IMDM medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies), using RNeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45  $\mu$ l of sterile water, and 1  $\mu$ l of RQ1 RNase-Free DNase (manufactured by Promega), 5  $\mu$ l of the attached 10  $\times$  DNase buffer and 0.5  $\mu$ l of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction,

the total RNA was purified again using RNAeasy (manufactured by QIAGEN) and dissolved in 50 µl of sterile water. In a 20 µl reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 3 µg of the obtained total RNA by reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions.

(0264)

Mouse FUT8 cDNA was prepared by the following procedure (Fig. 17).

First, a forward primer specific for a sequence containing a translation initiation codon (shown in SEQ ID NO:19) and a reverse primer specific for a sequence containing translation termination codon (shown in SEQ ID NO:20) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25 µl of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5 µmol/l specific primers (SEQ ID NO:19 and SEQ ID NO:20)] containing 1 µl of the NSO cell-derived cDNA was prepared, and PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0265)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of 1728 bp was purified. Into a plasmid pCR2.1, 4 µl of the DNA fragment was employed to insert in accordance with the manufacturer's instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5α was transformed with the reaction solution.

Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 6 clones among the obtained kanamycin-resistant colonies.

(0266)

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed that all of the inserted cDNAs of which sequences were determined encode the ORF full sequence of mouse FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences were selected (its DNA sequence and amino acid sequence are shown in SEQ ID NOs:2 and 24, respectively). Also, inconsistency of 3 bases due to amino acid substitution was found in the sequence when compared with the mouse FUT8 sequence registered on GenBank. Herein, the plasmid is referred to mfFUT8-pCR2.1.

(0267)

Next, a plasmid pBSmfFUT8 containing the ORF full sequence of mouse FUT8 was constructed as follows (Fig. 18). First, 1 µg of a plasmid pBluescript II KS(+) (manufactured by Stratagene) was dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. To the reaction solution, 35 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 µl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added, followed by reaction at 65°C for 30 minutes for dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform, followed by ethanol precipitated, and the recovered DNA fragment was dissolved in 10 µl of sterile water.

(0268)

Separately, 1 µg of the plasmid mfFUT8-pCR2.1 was dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo) were added thereto, followed by digestion reaction at 37°C for 2 hours. The reaction solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

The obtained plasmid pBluescript II KS(+)-derived *EcoRI-EcoRI* fragment (1 µl, 2.9 Kb), 4 µl of the *EcoRI-EcoRI* fragment (1.7 Kb) prepared from the plasmid mfFUT8-pCR2.1 and 5 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and plasmid DNAs were isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pBSmfFUT8.

(0269)

Using the pBSmfFUT8 and pAGE249, a mouse FUT8 expression vector pAGEmfFUT8 was constructed by the following procedure (Fig. 19). The pAGE249 is a derivative of pAGE248 [*J. Biol. Chem.*, 269, 14730 (1994)], as a vector in which an *SphI-SphI* fragment (2.7 Kb) containing a dihydrofolate reductase gene (*dhfr*) expression unit was removed from the pAGE248.

In 50 µl of Universal Buffer H (manufactured by Takara Shuzo), 1 µg of the pAGE249 was dissolved, and 20 units of a restriction enzyme *SalI* (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *BamHI* (manufactured by New England Biolabs) were added

thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, to the reaction solution, 35 µl of 1 mol/l Tris-HCl buffer (pH 8.0) and 3.5 µl of *E. coli* C15-derived alkaline phosphatase (manufactured by Takara Shuzo) were added thereto, followed by reaction at 65°C for 30 minutes to dephosphorylate the DNA termini. The reaction solution was extracted with phenol/chloroform extraction, followed by ethanol precipitation, and the recovered DNA fragment was dissolved in 10 µl of sterile water.

(0270)

Separately, 1 µg of pBSmfFUT8 was dissolved in 50 µl of Universal Buffer H (manufactured by Takara Shuzo), and 20 units of a restriction enzyme *Sal*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. A DNA fragment was recovered from the reaction solution by ethanol precipitation and dissolved in 35 µl of NEBuffer 2 (manufactured by New England Biolabs), and 20 units of a restriction enzyme *Bam*HI (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours. After the digestion reaction, the solution was subjected to 0.8% agarose gel electrophoresis to purify a DNA fragment of about 1.7 Kb containing the ORF full sequence of mouse FUT8 cDNA.

(0271)

The obtained plasmid pAGE249-derived *Bam*HI-*Sal*I fragment (1 µl, 6.5 Kb), 4 µl of the *Bam*HI-*Sal*I fragment (1.7 Kb) prepared from the plasmid pBSmfFUT8 and 5 µl of Ligation High (manufactured by Toyobo) were mixed, followed by ligation reaction at 16°C for 30 minutes. Using the reaction solution, *E. coli* DH5α was transformed, and a plasmid DNA was isolated in accordance with a known method from the obtained ampicillin-resistant clones. Herein, the plasmid is referred to pAGEMfFUT8.

(0272)

(2) Preparation of mouse  $\alpha$ -1,6-fucosyltransferase (FUT8) gene over-expressing cell line

A stable FUT8 gene-expressing cell line was obtained by introducing the mouse FUT8 expression vector pAGEmfFUT8 constructed in the item (1) into 61-33. The 61-33 is a clone obtained by carrying out serum-free adaptation of a transformant cell 7-9-51 (FERM BP-6691, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology) derived from a YB2/0 cell highly producing an anti-ganglioside GD3 chimeric antibody, and then carrying out single cell isolation by two limiting dilution.

(0273)

The plasmid pAGEmfFUT8 was transferred into 61-33 by the following procedure in accordance with the electroporation [Cytotechnology, 3, 133 (1990)]. First, 30  $\mu$ g of the plasmid pAGEmfFUT8 was dissolved in 600  $\mu$ l of NEBuffer 4 (manufactured by New England Biolabs), and 100 units of a restriction enzyme *Fsp*I (manufactured by New England Biolabs) were added thereto, followed by digestion reaction at 37°C for 2 hours to obtain a linear fragment. The reaction solution was subjected to ethanol precipitation, and the recovered linear plasmid was made into a 1  $\mu$ g/ $\mu$ l aqueous solution. Next, the 61-33 was suspended in a K-PBS buffer (137 mol/l KCl, 2.7 mol/l NaCl, 8.1 mol/l  $\text{Na}_2\text{HPO}_4$ , 1.5 mol/l  $\text{KH}_2\text{PO}_4$ , 4.0 mol/l  $\text{MgCl}_2$ ) to give a density of  $2 \times 10^7$  cells/ml, and 200  $\mu$ l of the cell suspension ( $4 \times 10^6$  cells) was mixed with 10  $\mu$ l (10  $\mu$ g) of the linear plasmid. The cell-DNA mixture was transferred into Gene Pulser Cuvette (inter-electrode distance, 2 mm) (manufactured by BIO-RAD) and then electroporation was carried out using a cell fusion apparatus Gene Pulser (manufactured by BIO-RAD) at 0.2 KV pulse voltage and 250  $\mu$ F electric capacity. The cell suspension was mixed with

10 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and dispensed in 100  $\mu$ l portions into a 96 well plate for suspension cell use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO<sub>2</sub>, 50  $\mu$ l of the culture supernatant was removed, and Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) was dispensed at 100  $\mu$ l. They were cultured for 3 weeks while repeating the medium exchange step at intervals of 3 to 4 days, and 14 cell lines showing hygromycin resistance were obtained.

(0274)

On the other hand, a negative control cell line was prepared by introducing the plasmid pAGE249 as a parent vector of the pAGEmfFUT8 into the 61-33. According to the above procedure, 10  $\mu$ g of the plasmid pAGE249 converted into linear form with a restriction enzyme *FspI* was introduced into  $4 \times 10^6$  cells of the 61-33 using the electroporation. The cells were mixed with 15 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies), transferred into a T75 flask for suspension cell (manufactured by Greiner) and then cultured at 37°C for 24 hours in 5% CO<sub>2</sub>. After culturing them, a half of the culture supernatant (7.5 ml) was removed by centrifugation at 800 rpm for 4 minutes, and the cells were suspended in 7.5 ml of Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical

Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) and transferred into the T75 flask for suspension cell (manufactured by Greiner). They were cultured for 3 weeks while repeating the medium exchange at intervals of 3 to 4 days, a hygromycin-resistant cell line was obtained.

(0275)

(3) Analysis of expression level of  $\alpha$ -1,6-fucosyltransferase (FUT8) gene in cell lines over-expressing the gene

Using 6 cell lines optionally selected from the 14 mouse FUT8-over expressing cell lines prepared from 61-33 in the item (2) and the negative control cell line, the FUT8 expression levels were compared using competitive RT-PCR.

Each of these over-expression cell lines was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries), 5% fetal bovine dialyzed serum (manufactured by Life Technologies) and 0.2% BSA (manufactured by Life Technologies) to give a density of  $3 \times 10^5$  cells/ml and then transferred into a T75 flask for suspension cell culture use (manufactured by Greiner). After culturing them at 37°C for 24 hours in 5% CO<sub>2</sub>,  $1 \times 10^7$  of intact cells were recovered to extract total RNA using RNAeasy (manufactured by QIAGEN) in accordance with the manufacture's instructions. The total RNA was dissolved in 45  $\mu$ l of sterile water, and 0.5 U/ $\mu$ l of RQ1 RNase-Free DNase (manufactured by Promega), 5  $\mu$ l of the attached 10  $\times$  DNase buffer and 0.5  $\mu$ l of RNasin Ribonuclease Inhibitor (manufactured by Promega) were added thereto, followed by reaction at 37°C for 30 minutes to degrade genome DNA contaminated in the sample. After the reaction, the total RNA was purified again using RNAeasy

(manufactured by QIAGEN) and dissolved in 50  $\mu$ l of sterile water.

(0276)

In a 20  $\mu$ l reaction mixture using oligo(dT) as a primer, single-stranded cDNA was synthesized from 2.5  $\mu$ g of the obtained total RNA by reverse transcription reaction using SUPERScript™ Preamplification System for First Strand cDNA Synthesis (manufactured by Life Technologies) in accordance with the manufacture's instructions. The reaction solution was diluted 50 folds with water and the transcription amount of each gene was determined by the competitive PCR in accordance with Example 8(6).

The FUT8 gene-derived mRNA transcription amount in each expression cell line was determined by the following procedure.

YBFT8-pCR2.1, as a plasmid in which a cDNA partial fragment prepared in Example 8(2) from rat FUT8 was inserted into pCR2.1, was digested with a restriction enzyme *EcoRI*, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the FUT8 transcription amount.

(0277)

Among the YBFT8-pCR2.1 prepared in Example 8(4), YBFT8d-pCR2.1 obtained by deleting 203 bp between *ScaI* and *HindIII* of an inner nucleotide sequence of rat FUT8 was digested with a restriction enzyme *EcoRI*, and the obtained linear DNA was used as the internal control for FUT8 determination.

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20  $\mu$ l in total volume of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l FUT8 gene-specific primers (SEQ ID NOs:13 and 14) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from respective expression cell line in the above and 5  $\mu$ l (10 fg) of the

plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 32 cycles of heating at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute as one cycle.

(0278)

Also, PCR was carried out in a series of reaction in which 5 µl (0.1 fg, 1 fg, 5 fg, 10 fg, 50 fg, 100 fg, 500 fg or 1 pg) of the FUT8 standard plasmid was added instead of the each expression cell line-derived cDNA, and used in the preparation of a calibration curve for the FUT8 transcription amount. In this case, 1 µg/ml baker's yeast t-RNA (manufactured by SIGMA) was used for the dilution of standard plasmid.

On the other hand, since it is considered that the  $\beta$ -actin gene is transcribed constantly in each cell and its transcription level is approximately the same between cells, the transcription amount of the  $\beta$ -actin gene was determined as an index of the efficiency of synthesis reaction of cDNA in each expression cell line.

YBac-pBS, as a plasmid in which the ORF full sequence of cDNA of rat  $\beta$ -actin was inserted into pBluescript II KS(+) prepared in Example 8(3), was digested with restriction enzymes *Hind*III and *Kpn*I, and the obtained linear DNA was used as the standard in the preparation of a calibration curve for determining the  $\beta$ -actin gene transcription amount.

YBacd-pBS obtained from the YBac-pBS prepared in Example 8(5) by deleting 180 bp between *Dra*I and *Dra*I of an inner nucleotide sequence of rat  $\beta$ -actin was digested with restriction enzymes *Hind*III and *Kpn*I, and the obtained linear DNA was used as the internal standards for  $\beta$ -actin amount determination.

(0279)

PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo) in 20 µl in total volume of

a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 0.5  $\mu$ mol/l  $\beta$ -actin-specific primers (SEQ ID NOs:17 and 18) and 5% DMSO] containing 5  $\mu$ l of 50 folds-diluted cDNA solution prepared from each of the expression cell lines and 5  $\mu$ l (1 pg) of the plasmid for internal control. The PCR was carried out by heating at 94°C for 3 minutes and subsequent 17 cycles of heating at 94°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes as one cycle.

Also, PCR was carried out in a series of reaction in which 10 pg, 5 pg, 1 pg, 500 fg or 100 fg of the  $\beta$ -actin standard plasmid was added instead of the each expression cell line-derived cDNA, and used in the preparation of a calibration curve for the  $\beta$ -actin transcription amount. In this case, 1  $\mu$ g/ml baker's yeast t-RNA (manufactured by SIGMA) was used for diluting the standard plasmid.

(0280)

By carrying out PCR using the primer set described in Table 3, a DNA fragment having a size shown in the target column of Table 3 can be amplified from each gene transcription product and each standard, and a DNA fragment having a size shown in the competitor column of Table 3 can be amplified from each internal control.

Each (7  $\mu$ l) of the solutions after PCR was subjected to a 1.75% agarose gel electrophoresis, and then the gel was stained by soaking it for 30 minutes in 1  $\times$  concentration SYBR Green I Nucleic Acid Gel Stain (manufactured by Molecular Probes). By calculating luminescence intensity of each amplified DNA fragment using a fluoro-imager (FluorImager SI; manufactured by Molecular Dynamics), the amount of the amplified DNA fragment was measured.

The amount of an amplified product formed by PCR using the standard plasmid as the template was measured by the method, and a calibration curve was prepared by

plotting the measured values against the amounts of the standard plasmid. Using the calibration curve, the amount of cDNA of a gene of interest in each cell line was calculated from the amount of an amplified product when each expression cell line-derived cDNA was used as the template, and the amount was defined as the mRNA transcription amount in each cell line.

(0281)

Fig. 20 shows the FUT8 transcription amounts as relative values to the amount of  $\beta$ -actin transcription product. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 and the pAGE249-introduced cell line were cell lines having a relatively small FUT8 transcription amount, which was equivalent to 0.3 to 10% of a  $\beta$ -actin transcription amount. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 were cell lines having a relatively large FUT8 transcription amount, which was equivalent to 20 to 40% of a  $\beta$ -actin transcription amount.

(0282)

(4) Purification of antibody produced by  $\alpha$ -1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Each of the six FUT8 gene over-expressing cell lines and one negative control cell line obtained in the item (2) was suspended in Hybridoma-SFM medium (manufactured by Life Technologies) supplemented with 200 nmol/l MTX, 0.5 mg/ml Hygromycin B (manufactured by Wako Pure Chemical Industries) and 0.2% BSA (manufactured by Life Technologies) to give a density of  $2 \times 10^5$  cells/ml, and then 100 ml in total of the suspension was inoculated into three T225 flasks for suspension cell culture use (manufactured by IWAKI). After culturing them at 37°C for 7 to 9 days in a 5% CO<sub>2</sub> incubator, the number of intact cells was counted to confirm that their viability was almost the same (each 30% or less), and then each cell

suspension was recovered. Each of the cell suspensions was centrifuged at 3,000 rpm at 4°C for 10 minutes, and the recovered supernatant was centrifuged at 10,000 rpm at 4°C for 1 hour and then filtered using PES Filter Unit (manufactured by NALGENE) having a pore diameter of 0.22 µm with 150 ml capacity.

(0283)

Prosep-A HighCapacity (manufactured by bioPROCESSING) was packed in a 0.8 cm diameter column to a thickness of 2 cm and washed with 10 ml of 0.1 mol/l citrate buffer (pH 3.0) and 10 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6) in that order to effect equilibration the carrier. Next, 100 ml of each of the culture supernatant was passed through the column and washed with 50 ml of 1 mol/l glycine/NaOH-0.15 mol/l NaCl buffer (pH 8.6). After washing them, the antibody absorbed to Prosep-A was eluted using 2.5 ml of a 0.1 mol/l citrate buffer (pH 3.0), the eluate was fractionated at 500 µl and each fraction was neutralized by mixing with 100 µl of 2 mol/l Tris-HCl (pH 8.5). Two fractions containing the antibody at a high concentration (1.2 ml in total) were selected by the BCA method [*Anal. Biochem.*, 150, 76 (1985)], combined and then dialyzed against 10 mol/l citrate buffer (pH 6.0) at 4°C for a whole day and night. After the dialysis, the antibody solution was recovered and subjected to sterile filtration using a 0.22 µm pore size Millex GV (manufactured by MILLIPORE).

(0284)

(5) *In vitro* cytotoxic activity (ADCC activity) of antibody produced by mouse  $\alpha$ -1,6-fucosyltransferase (FUT8) gene over-expressing cell line

In order to evaluate *in vitro* cytotoxic activity of the anti-GD3 antibodies purified in the item (4), ADCC activity was measured using a GD3-positive cell, human

melanoma cultured cell line G-361 (RCB0991, Cell Bank at The Institute of Physical and Chemical Research).

The G-361 cells subcultured in RPMI1640 medium (manufactured by Life Technologies) containing 10% fetal bovine serum (manufactured by Life Technologies) (hereinafter referred to as "RPMI1640-FBS(10)") were suspended in 500  $\mu$ l of RPMI1640-FBS(10) at a density of  $1 \times 10^6$  cells, and 3.7 MBq of  $\text{Na}_2^{51}\text{CrO}_4$  was added thereto, followed by culturing at 37°C for 30 minutes for labeling the cells with a radioisotope. After centrifugation at 1,200 rpm for 5 minutes, the supernatant was discarded and the target cells were suspended in 5 ml of RPMI1640-FBS(10). The washing step was repeated three times and then the cell suspension was incubated for 30 minutes on ice for spontaneous dissociation of the radioactive substance. The washing step was again repeated twice and then the cells were suspended in 5 ml of RPMI1640-FBS(10) to thereby prepare  $2 \times 10^5$  cells/ml of a target cell suspension.  
(0285)

On the other hand, 30 ml of peripheral blood was collected from a healthy person and gently mixed with 0.5 ml of heparin sodium (manufactured by Shimizu Pharmaceutical) and then mixed with 30 ml of physiological saline (manufactured by Otsuka Pharmaceutical). After the mixing, 10 ml of the mixture was gently overlaid on 4 ml of Lymphoprep (manufactured by NYCOMED PHARMA AS) and centrifuged at room temperature at 2,000 rpm for 30 minutes. The separated mononuclear cell fractions were collected from the centrifugation tubes, combined and then suspended in 30 ml of RPMI1640-FBS(10). After centrifugation at room temperature at 1,200 rpm for 5 minutes, the supernatant was discarded and the cells were suspended in 20 ml of RPMI1640-FBS(10). The washing step was repeated twice and then  $2 \times 10^6$  cells/ml of an effector cell suspension was prepared using RPMI1640-FBS(10).

(0286)

The target cell suspension was dispensed at 50  $\mu$ l ( $1 \times 10^4$  cells/well) into each well of a 96 well U-bottom plate (manufactured by Falcon). Subsequently, the effector cell suspension was dispensed at 100  $\mu$ l ( $2 \times 10^5$  cells/well) into each well to thereby adjust the ratio of the effector cells to the target cells to 20 : 1. Next, using a 10 M citrate buffer (pH 6.0), a series of dilution solution of 0.01  $\mu$ g/ml, 0.1  $\mu$ g/ml, 1  $\mu$ g/ml and 10  $\mu$ g/ml of each anti-GD3 antibody obtained in the item (4) was prepared, and the diluted solutions were dispensed at 50  $\mu$ l into the wells to give final concentrations of 0.0025  $\mu$ g/ml, 0.025  $\mu$ g/ml, 0.25  $\mu$ g/ml and 2.5  $\mu$ g/ml, respectively. After carrying out the reaction at 37°C for 4 hours in 5% CO<sub>2</sub>, the plate was centrifuged at 1,200 rpm for 5 minutes. Into a 12 mm diameter RIA tube (manufactured by IWAKI), 50  $\mu$ l of the supernatant in each well was transferred and, and the amount of the dissociated <sup>51</sup>Cr was measured using MINAX- $\gamma$  auto-gamma counter 5550 (manufactured by PACKARD).

(0287)

Also, the amount of the spontaneously dissociated <sup>51</sup>Cr was calculated by carrying out the same reaction in a reaction mixture in which 150  $\mu$ l of RPMI1640-FBS(10) was added instead of the effector cell suspension and antibody solution. The amount of the total dissociated <sup>51</sup>Cr was calculated by carrying out the same reaction in a reaction mixture in which 100  $\mu$ l of 1 N hydrochloric acid and 50  $\mu$ l of RPMI1640-FBS(10) were added instead of the effector cell suspension and antibody solution. Using these values, the ADCC activity was calculated based on the formula (II) described in the item 2(3) of Example 2.

(0288)

Fig. 21 shows ADCC activity of each of the anti-GD3 antibodies for G-361 cell. Three cell lines mfFUT8-1, mfFUT8-2 and mfFUT8-4 having a low FUT8 expression level as

shown in Fig. 20 showed potent ADCC activity equivalent to that of the negative control pAGE249-introduced cell line. On the other hand, other three cell lines mfFUT8-3, mfFUT8-6 and mfFUT8-7 having a high FUT8 expression level as shown in Fig. 20 showed low ADCC activity equivalent to that of the anti-GD3 antibody produced from CHO cell. Based on these results, it was shown that the ADCC activity of produced antibodies can be controlled by regulating the expression level of FUT8 in host cells.

(0289)

(6) Sugar chain analysis of antibody produced by mouse  $\alpha$ -1,6-fucosyltransferase (FUT8) gene over-expressing cell line

Sugar chains of the anti-GD3 antibodies purified in the item (4) were analyzed. The sugar chains binding to the antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines were cleaved from proteins by subjecting the antibodies to hydrazinolysis [*Method of Enzymology*, **83**, 263 (1982)]. After removing hydrazine by evaporation under a reduced pressure, *N*-acetylation was carried out by adding an aqueous ammonium acetate solution and acetic anhydride. After freeze-drying, fluorescence labeling by 2-aminopyridine [*J. Biochem.*, **95**, 197 (1984)] was carrying out. A fluorescence-labeled sugar chain group (PA-treated sugar chain group) was separated from excess reagents using Superdex Peptide HR 10/30 column (manufactured by Pharmacia). The sugar chain fractions were dried using a centrifugation concentrator and used as a purified PA-treated sugar chain group. Next, the purified PA-treated sugar chain group was subjected to reverse phase HPLC analysis using a CLC-ODS column (manufactured by Shimadzu) (Fig. 23). When calculated from the peak area, the content of  $\alpha$ -1,6-fucose-free sugar chains in mfFUT8-6 was 10%, and the content of  $\alpha$ -1,6-fucose-bound sugar chains was 90%. The content of  $\alpha$ -1,6-fucose-free sugar chains in pAGE249

was 20%, and the content of  $\alpha$ -1,6-fucose-bound sugar chains was 80%. Based on these results, it was found that the content of  $\alpha$ -1,6-fucose-bound sugar chains of a produced antibody is increased by over-expressing the FUT8 gene.

Fig. 23 shows elution patterns obtained by carrying out reverse phase HPLC analysis of each of PA-treated sugar chains prepared from antibodies produced by mfFUT8-6 and pAGE249-introduced cell lines. Figs. 23 (upper) and 23 (lower) show elution patterns of mfFUT8-6 and pAGE249, respectively. The relative fluorescence intensity and the elution time are plotted as the ordinate and the abscissa, respectively. Using a sodium phosphate buffer (pH 3.8) as buffer A and a sodium phosphate buffer (pH 3.8) + 0.5% 1-butanol as buffer B, the analysis was carried out by the following gradient.

Time (minute)	0	80	90	90.1	120
Buffer B (%)	0	60	60	0	0

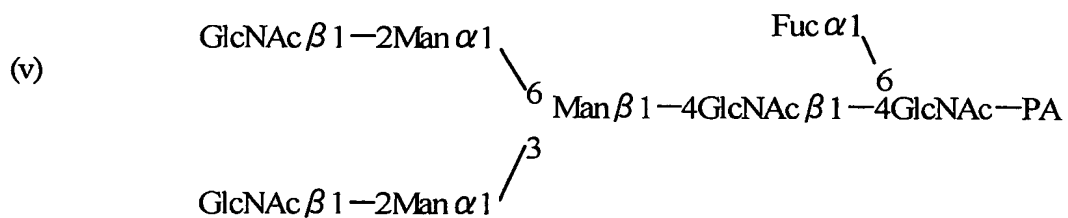
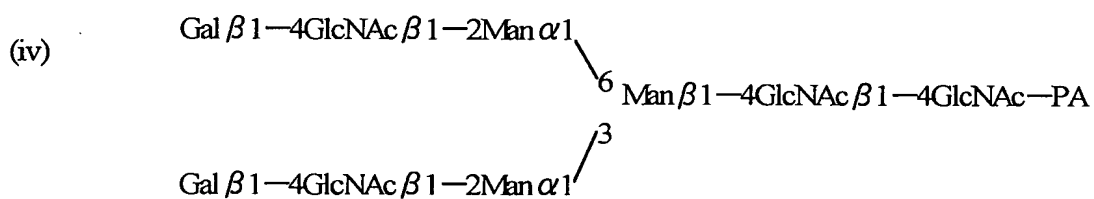
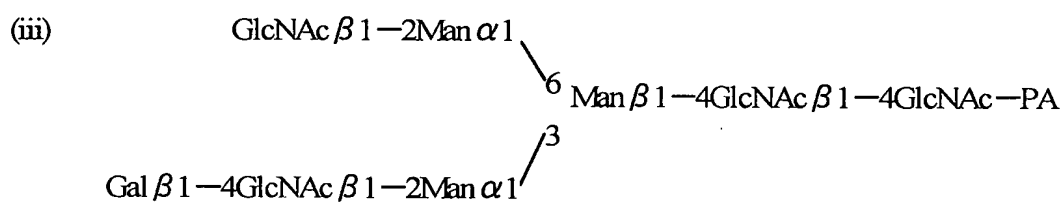
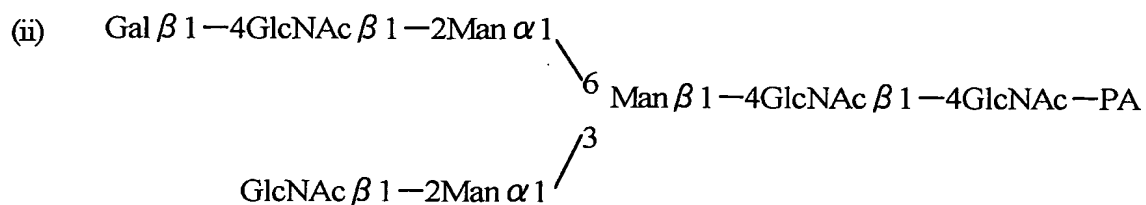
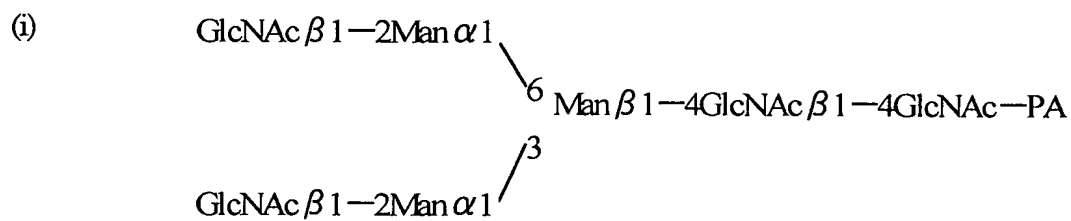
Peaks (i) to (ix) shown in Fig. 23 and Fig. 24 show the following structures.

(0290)

(Chem. 3)

(0291)

(Chem. 4)





(0292)

GlcNAc, Gal, Man, Fuc and PA indicate N-acetylglucosamine, galactose, mannose, fucose and a pyridylamino group, respectively. In Figs. 23 and 24, the ratio of the  $\alpha$ -1,6-fucose-free sugar chain group was calculated from the area occupied by the peaks (i) to (iv) among (i) to (ix), and the ratio of the  $\alpha$ -1,6-fucose-bound sugar chain group from the area occupied by the peaks (v) to (ix) among (i) to (ix).

(0293)

#### Example 11

Preparation of CHO cell  $\alpha$ -1,6-fucosyltransferase (FUT8) gene:

(1) Preparation of CHO cell  $\alpha$ -1,6-fucosyltransferase (FUT8) cDNA sequence

From a single-stranded cDNA prepared from CHO/DG44 cells on the 2nd day of culturing in Example 8(1), Chinese hamster FUT8 cDNA was obtained by the following procedure (Fig. 22).

First, a forward primer specific for a 5'-terminal non-translation region (shown in SEQ ID NO:21) and a reverse primer specific for a 3'-terminal non-translation region (shown in SEQ ID NO:22) were designed from a mouse FUT8 cDNA sequence (GenBank, AB025198).

Next, 25  $\mu$ l of a reaction solution [ExTaq buffer (manufactured by Takara Shuzo), 0.2 mmol/l dNTPs, 4% DMSO and 0.5  $\mu$ mol/l specific primers (SEQ ID NOs:21 and 22)] containing 1  $\mu$ l of the CHO/DG44 cell-derived cDNA was prepared and PCR was carried out using a DNA polymerase ExTaq (manufactured by Takara Shuzo). The PCR was carried out by heating at 94°C for 1 minute, subsequent 30 cycles of heating at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes as one cycle, and final heating at 72°C for 10 minutes.

(0294)

After the PCR, the reaction solution was subjected to 0.8% agarose gel electrophoresis, and a specific amplified fragment of about 2 Kb was purified. Into a plasmid pCR2.1, 4  $\mu$ l of the DNA fragment was employed to insert in accordance with the instructions attached to TOPO TA Cloning Kit (manufactured by Invitrogen), and *E. coli* DH5 $\alpha$  was transformed with the reaction solution. Plasmid DNAs were isolated in accordance with a known method from cDNA-inserted 8 clones among the obtained kanamycin-resistant colonies.

The nucleotide sequence of each cDNA inserted into the plasmid was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. It was confirmed by the method that all of the inserted cDNAs encode a sequence containing the full ORF of CHO cell FUT8. Among these, a plasmid DNA containing absolutely no reading error of bases by the PCR in the sequences was selected. Herein, the plasmid is referred to as CHfFUT8-pCR2.1. The determined nucleotide sequence and the amino acid sequence of the cDNA of CHO FUT8 are shown in SEQ ID NOs:1 and 23, respectively.

(0295)

(2) Preparation of CHO cell  $\alpha$ -1,6-fucosyltransferase (FUT8) genomic sequence

Using the ORF full length cDNA fragment of CHO cell FUT8 obtained in the item (1) as a probe, a CHO cell FUT8 genomic clone was obtained in accordance with a known genome screening method described, e.g., in *Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, A Laboratory Manual, Second Edition* (1989). Next, after digesting the obtained genomic clone using various restriction enzymes, the Southern hybridization was carried out using an AfaI-Sau3AI fragment (about 280 bp) containing

initiation codon of the CHO cell FUT8 cDNA as a probe, and then a XbaI-XbaI fragment (about 2.5 Kb) and a SacI-SacI fragment (about 6.5 Kb) were selected from restriction enzyme fragments showing positive reaction, inserted into pBluescript II KS(+) (manufactured by Stratagene), respectively.

(0296)

The nucleotide sequence of each of the obtained genomic fragments was determined using DNA Sequencer 377 (manufactured by Parkin Elmer) and BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by Parkin Elmer) in accordance with the method of the manufacture's instructions. Thereby, it was confirmed that the XbaI-XbaI fragment encodes a sequence of an upstream intron of about 2.5 Kb containing exon 2 of the CHO cell FUT8, and the SacI-SacI fragment encodes a sequence of a downstream intron of about 6.5 Kb containing exon 2 of the CHO cell FUT8. Herein, the plasmid containing XbaI-XbaI fragment is referred to as pFUT8fgE2-2, and the plasmid containing SacI-SacI fragment is referred to as pFUT8fgE2-4. The determined nucleotide sequence (about 9.0 Kb) of the genome region containing exon 2 of the CHO cell FUT8 is shown in SEQ ID NO:3.

(0297)

#### Example 12

Sugar chain analysis of conventionally available antibodies:

Sugar chains binding to a conventionally available anti-HER2/neu antibody Herceptin (manufactured by GENENTECH and Roche) produced by CHO cell as the host cell was analyzed in accordance with the method of Example 10(6) (Fig. 24). When calculated from each peak area of elution diagram, the content of  $\alpha$ -1,6-fucose-free sugar chains of Herceptin was 16%, and the content of  $\alpha$ -1,6-fucose-bound sugar chains was 84%. The same analysis was carried out on

other commercially available antibodies, Rituxan (manufactured by GENENTECH, Roche and IDEC) and Zenapax (manufactured by Roche and PDL), and the  $\alpha$ -1,6-fucose-free sugar chain content of was less than that in Herceptin.

Fig. 24 is a graph showing elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The relative fluorescence intensity and the elution time are plotted as the ordinate and the abscissa, respectively. The reverse phase HPLC analysis conditions, sugar chain structure analysis and calculation of the ratio of sugar chain group containing no  $\alpha$ -1,6-fucose sugar chain were carried out.

(0298)

(Effect of the invention)

The present invention provides a cell for the production of a glycoprotein such as an immunologically functional molecule or the like, a method for producing an immunologically functional molecule using the cell, an immunologically functional molecule, and use thereof.

(0299)

(Sequence listing free text)

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SEQ ID NO:5- Explanation of artificial sequence: primer sequence of artificially synthesized primer sequences

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SEQ ID NO:10- Explanation of artificial sequence: primer sequence of artificially synthesized primer sequences

SEQ ID NO:11- Explanation of artificial sequence: primer sequence of artificially synthesized primer sequences

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(Sequence listing)

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<400> 18  
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<400> 19  
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<400> 20  
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 Asn Asp His Pro Asp His Ser Ser Arg Glu Leu Ser Lys Ile Leu Ala  
 35 40 45  
 Lys Leu Glu Arg Leu Lys Gln Gln Asn Glu Asp Leu Arg Arg Met Ala  
 50 55 60  
 Glu Ser Leu Arg Ile Pro Glu Gly Pro Ile Asp Gln Gly Thr Ala Thr  
 65 70 75 80  
 Gly Arg Val Arg Val Leu Glu Glu Gln Leu Val Lys Ala Lys Glu Gln  
 85 90 95  
 Ile Glu Asn Tyr Lys Lys Gln Ala Arg Asn Asp Leu Gly Lys Asp His  
 100 105 110

Glu Ile Leu Arg Arg Arg Ile Glu Asn Gly Ala Lys Glu Leu Trp Phe  
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 Phe Leu Gln Ser Glu Leu Lys Lys Leu Lys Lys Leu Glu Gly Asn Glu  
           130                          135                          140  
 Leu Gln Arg His Ala Asp Glu Ile Leu Leu Asp Leu Gly His His Glu  
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 Arg Ser Ile Met Thr Asp Leu Tyr Tyr Leu Ser Gln Thr Asp Gly Ala  
                           165                          170                          175  
 Gly Glu Trp Arg Glu Lys Glu Ala Lys Asp Leu Thr Glu Leu Val Gln  
                           180                          185                          190  
 Arg Arg Ile Thr Tyr Leu Gln Asn Pro Lys Asp Cys Ser Lys Ala Arg  
                           195                          200                          205  
 Lys Leu Val Cys Asn Ile Asn Lys Gly Cys Gly Tyr Gly Cys Gln Leu  
           210                          215                          220  
 His His Val Val Tyr Cys Phe Met Ile Ala Tyr Gly Thr Gln Arg Thr  
   225                          230                          235                          240  
 Leu Ile Leu Glu Ser Gln Asn Trp Arg Tyr Ala Thr Gly Gly Trp Glu  
                           245                          250                          255  
 Thr Val Phe Arg Pro Val Ser Glu Thr Cys Thr Asp Arg Ser Gly Leu  
                           260                          265                          270  
 Ser Thr Gly His Trp Ser Gly Glu Val Lys Asp Lys Asn Val Gln Val  
           275                          280                          285  
 Val Glu Leu Pro Ile Val Asp Ser Leu His Pro Arg Pro Pro Tyr Leu  
   290                          295                          300  
 Pro Leu Ala Val Pro Glu Asp Leu Ala Asp Arg Leu Leu Arg Val His  
   305                          310                          315                          320  
 Gly Asp Pro Ala Val Trp Trp Val Ser Gln Phe Val Lys Tyr Leu Ile  
                           325                          330                          335  
 Arg Pro Gln Pro Trp Leu Glu Arg Glu Ile Glu Glu Thr Thr Lys Lys  
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 Leu Gly Phe Lys His Pro Val Ile Gly Val His Val Arg Arg Thr Asp  
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 Lys Val Gly Thr Glu Ala Ala Phe His Pro Ile Glu Glu Tyr Met Val  
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 His Val Glu Glu His Phe Gln Leu Leu Glu Arg Arg Met Lys Val Asp  
   385                          390                          395                          400  
 Lys Lys Arg Val Tyr Leu Ala Thr Asp Asp Pro Ser Leu Leu Lys Glu  
                           405                          410                          415  
 Ala Lys Thr Lys Tyr Ser Asn Tyr Glu Phe Ile Ser Asp Asn Ser Ile  
                           420                          425                          430  
 Ser Trp Ser Ala Gly Leu His Asn Arg Tyr Thr Glu Asn Ser Leu Arg  
           435                          440                          445

Gly Val Ile Leu Asp Ile His Phe Leu Ser Gln Ala Asp Phe Leu Val  
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 Cys Thr Phe Ser Ser Gln Val Cys Arg Val Ala Tyr Glu Ile Met Gln  
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 Thr Leu His Pro Asp Ala Ser Ala Asn Phe His Ser Leu Asp Asp Ile  
 485 490 495  
 Tyr Tyr Phe Gly Gly Gln Asn Ala His Asn Gln Ile Ala Val Tyr Pro  
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<210> 24  
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 50 55 60  
 Glu Ser Leu Arg Ile Pro Glu Gly Pro Ile Asp Gln Gly Thr Ala Thr  
 65 70 75 80  
 Gly Arg Val Arg Val Leu Glu Glu Gln Leu Val Lys Ala Lys Glu Gln  
 85 90 95  
 Ile Glu Asn Tyr Lys Lys Gln Ala Arg Asn Gly Leu Gly Lys Asp His  
 100 105 110  
 Glu Ile Leu Arg Arg Arg Ile Glu Asn Gly Ala Lys Glu Leu Trp Phe  
 115 120 125  
 Phe Leu Gln Ser Glu Leu Lys Lys Leu Lys His Leu Glu Gly Asn Glu  
 130 135 140  
 Leu Gln Arg His Ala Asp Glu Ile Leu Leu Asp Leu Gly His His Glu  
 145 150 155 160  
 Arg Ser Ile Met Thr Asp Leu Tyr Tyr Leu Ser Gln Thr Asp Gly Ala

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Gly	Asp	Trp	Arg	Glu	Lys	Glu	Ala	Lys	Asp	Leu	Thr	Glu	Leu	Val	Gln				
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Arg	Arg	Ile	Thr	Tyr	Leu	Gln	Asn	Pro	Lys	Asp	Cys	Ser	Lys	Ala	Arg				
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Lys	Leu	Val	Cys	Asn	Ile	Asn	Lys	Gly	Cys	Gly	Tyr	Gly	Cys	Gln	Leu				
	210					215					220								
His	His	Val	Val	Tyr	Cys	Phe	Met	Ile	Ala	Tyr	Gly	Thr	Gln	Arg	Thr				
225					230					235					240				
Leu	Ile	Leu	Glu	Ser	Gln	Asn	Trp	Arg	Tyr	Ala	Thr	Gly	Gly	Trp	Glu				
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Thr	Val	Phe	Arg	Pro	Val	Ser	Glu	Thr	Cys	Thr	Asp	Arg	Ser	Gly	Leu				
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Ser	Thr	Gly	His	Trp	Ser	Gly	Glu	Val	Asn	Asp	Lys	Asn	Ile	Gln	Val				
		275					280					285							
Val	Glu	Leu	Pro	Ile	Val	Asp	Ser	Leu	His	Pro	Arg	Pro	Pro	Tyr	Leu				
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Pro	Leu	Ala	Val	Pro	Glu	Asp	Leu	Ala	Asp	Arg	Leu	Leu	Arg	Val	His				
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Gly	Asp	Pro	Ala	Val	Trp	Trp	Val	Ser	Gln	Phe	Val	Lys	Tyr	Leu	Ile				
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Arg	Pro	Gln	Pro	Trp	Leu	Glu	Lys	Glu	Ile	Glu	Glu	Ala	Thr	Lys	Lys				
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Leu	Gly	Phe	Lys	His	Pro	Val	Ile	Gly	Val	His	Val	Arg	Arg	Thr	Asp				
		355					360					365							
Lys	Val	Gly	Thr	Glu	Ala	Ala	Phe	His	Pro	Ile	Glu	Glu	Tyr	Met	Val				
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His	Val	Glu	Glu	His	Phe	Gln	Leu	Leu	Ala	Arg	Arg	Met	Gln	Val	Asp				
385					390					395					400				
Lys	Lys	Arg	Val	Tyr	Leu	Ala	Thr	Asp	Asp	Pro	Thr	Leu	Leu	Lys	Glu				
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Ala	Lys	Thr	Lys	Tyr	Ser	Asn	Tyr	Glu	Phe	Ile	Ser	Asp	Asn	Ser	Ile				
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Ser	Trp	Ser	Ala	Gly	Leu	His	Asn	Arg	Tyr	Thr	Glu	Asn	Ser	Leu	Arg				
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	450					455					460								
Cys	Thr	Phe	Ser	Ser	Gln	Val	Cys	Arg	Val	Ala	Tyr	Glu	Ile	Met	Gln				
465					470					475					480				
Thr	Leu	His	Pro	Asp	Ala	Ser	Ala	Asn	Phe	His	Ser	Leu	Asp	Asp	Ile				
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Tyr	Tyr	Phe	Gly	Gly	Gln	Asn	Ala	His	Asn	Gln	Ile	Ala	Val	Tyr	Pro				



and the antibody concentration, respectively. "○", "●", "□", "■" and "△" show the activities of YB2/0-GD3 chimeric antibody, CHO/DG44-GD3 chimeric antibody, SP2/0-GD3 chimeric antibody, NS0-GD3 chimeric antibody (302) and NS0-GD3 chimeric antibody (GIT), respectively.  
(Fig. 4)

Fig. 4 shows electrophoresis patterns of SDS-PAGE of three purified anti-hIL-5R $\alpha$  CDR-grafted antibodies (using gradient gel from 4 to 15%). Fig. 4 (upper) and Fig. 4 (lower) show results of the electrophoresis carried out under non-reducing conditions and those under reducing conditions, respectively. Lanes 1 to 5 show electrophoresis patterns of high molecular weight markers, YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody, NS0-hIL-5R CDR antibody and low molecular weight markers, respectively.  
(Fig. 5)

Fig. 5 shows activities of three purified anti-hIL-5R $\alpha$  CDR-grafted antibodies to bind to hIL-5R $\alpha$ , measured by changing the antibody concentration. The ordinate and the abscissa show the binding activity with hIL-5R $\alpha$  and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5R CDR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.  
(Fig. 6)

Fig. 6 show ADCC activities of three purified anti-hIL-5R $\alpha$  CDR-grafted antibodies for an hIL-5R expressing mouse T cell line CTLL-2(h5R). The ordinate and the abscissas show the cytotoxic activity and the antibody concentration, respectively. "○", "●" and "□" show the activities of YB2/0-hIL-5RCR antibody, CHO/d-hIL-5R CDR antibody and NS0-hIL-5R CDR antibody, respectively.  
(Fig. 7)

Fig. 7 shows inhibition activities of three purified anti-hIL-5R $\alpha$  CDR-grafted antibodies in an hIL-5-induced eosinophil increasing model of *Macaca fascicularis*. The ordinate and the abscissa show the number of eosinophils in peripheral blood and the number of days (the day of the commencement of antibody and hIL-5 administration was defined as 0 day). "101 and 102", "301, 302 and 303", "401, 402 and 403" and "501, 502 and 503" show results in the antibody non-administration group, the YB2/0-hIL-5R CDR antibody administered group, the CHO/d-hIL-5R CDR antibody administered group and the NS0-hIL-5R CDR antibody administered group, respectively.

(Fig. 8)

Fig. 8 shows elution patterns of reverse phase HPLC elution of a PA-treated sugar chain (left side), and an elution pattern obtained by treating the PA-treated sugar chain with  $\alpha$ -L-fucosidase and then analyzed by reverse phase HPLC (right side), of the purified anti-hIL-5R $\alpha$  CDR-grafted antibody produced by YB2/0 (Fig. 8, upper) and the purified anti-hIL-5R $\alpha$  CDR-grafted antibody produced by NS0 (Fig. 8, lower). The ordinates and the abscissas show the relative fluorescence intensity and the elution time, respectively.

(Fig. 9)

Fig. 9 shows an elution pattern obtained by preparing a PA-treated sugar chain from the purified anti-hIL-5R $\alpha$  CDR-grafted antibody produced by CHO/d cell and analyzing it by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.

(Fig. 10)

In Fig. 10, Fig. 10 (upper) shows the GD3-binding activities of a non-adsorbed fraction and a part of an adsorbed fraction, measured by changing the antibody concentration. The ordinate and the abscissa show the

binding activity with GD3 and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively. Fig. 10 (lower) shows the ADCC activities of the non-adsorbed fraction and a part of the adsorbed fraction for a human melanoma line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively. "●" and "○" show the non-adsorbed fraction and a part of the adsorbed fraction, respectively.

(Fig. 11)

Fig. 11 shows elution patterns obtained by analyzing PA-treated sugar chains prepared from a non-adsorbed fraction and a part of an adsorbed fraction by a reverse HPLC. Fig. 11 (left) and Fig. 11 (right) show an elution pattern of the non-adsorbed fraction and an elution pattern of a part of the adsorbed fraction, respectively. The ordinates and the abscissas show the relative fluorescence strength and the elution time, respectively.

(Fig. 12)

Fig. 12 shows construction of plasmids CHFT8-pCR2.1 and YBFT8-pCR2.1.

(Fig. 13)

Fig. 13 shows construction of plasmids CHAc-pBS and YBAC-pBS.

(Fig. 14)

Fig. 14 shows construction of plasmids CHFT8d-pCR2.1 and YBFT8d-pCR2.1.

(Fig. 15)

Fig. 15 shows construction of plasmids CHAc-d-pBS and YBAC-d-pBS.

(Fig. 16)

Fig. 16 shows results of determination of an FUT8 transcription product in each host cell line using competitive RT-PCR. Amounts of the FUT8 transcription product in each host cell line when rat FUT8 sequence was

used as the standard and internal control are shown. "■" and "□" show results when CHO cell line and YB2/0 cell line, respectively, were used as the host cell.

(Fig. 17)

Fig. 17 shows construction of a plasmid mFUT8-pCR2.1.

(Fig. 18)

Fig. 18 shows construction of a plasmid pBSmFUT8.

(Fig. 19)

Fig. 19 shows construction of a plasmid pAGEmFUT8.

(Fig. 20)

Fig. 20 shows results of analysis of expression levels of FUT8 gene by a cell line excessively expressing the gene using a competitive RT-PCR. The ordinate shows relative values of amounts of FUT8 transcription to amounts of  $\beta$ -actin transcription.

(Fig. 21)

Fig. 21 shows ADCC activities of an anti-GD3 chimeric antibody purified from a cell line excessively expressing FUT8 gene against a human melanoma cell line G-361. The ordinate and the abscissa show the cytotoxic activity and the antibody concentration, respectively.

(Fig. 22)

Fig. 22 shows construction of a plasmid CHfFUT8-pCR2.1.

(Fig. 23)

Fig. 23 shows elution patterns of PA-treated sugar chains prepared from antibodies produced by mFUT8-6 and pAGE249-introduced cell lines, obtained by analyzing them by reverse phase HPLC. Fig. 30 (upper) and Fig. 30 (lower) show elution patterns of PA-treated sugar chains prepared from an antibody produced by mFUT8-6-introduced cell line and PA-treated sugar chains prepared from an antibody produced by pAGE249-introduced cell line, respectively.

The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively. (Fig. 24)

Fig. 24 shows an elution pattern of PA-treated sugar chains prepared from Herceptin, obtained by analyzing them by reverse phase HPLC. The ordinate and the abscissa show the relative fluorescence intensity and the elution time, respectively.